2004-05-24

The Role of γ_c Cytokines in T Cell Development, T Cell Homeostasis and CD8+ T Cell Function: A Dissertation

Sara Gozalo
University of Massachusetts Medical School

THE ROLE OF γ_C CYTOKINES IN T CELL DEVELOPMENT, T CELL HOMEOSTASIS AND CD8$^+$ T CELL FUNCTION

A Dissertation Presented

By

Sara Gozalo

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

MAY 24th, 2004

IMMUNOLOGY AND VIROLOGY PROGRAM

COPYRIGHT INFORMATION

The information and data in this thesis have appeared in the following publications:

Gozalo-Sanmillan S., McNally J.M., Lin M.Y., Chambers C.A.., Berg L.J. 2001. Two Distinct Mechanisms lead to Impaired T cell Homeostasis in Janus Kinase 3- and CTLA-4-Deficient Mice. *J. Immunol* **166**(2):727.

Gozalo-Sanmillan S., Brehm M.A., Welsh R.M., Berg L.J. 2004. Antiviral Immune Responses are Initiated but not Sustained by T Cells Lacking Jak3-Mediated Cytokine Signals. Manuscript to be submitted.

THE ROLE OF γ_C CYTOKINES IN T CELL DEVELOPMENT, T CELL HOMEOSTASIS AND $CD8^+$ T CELL FUNCTION

A Dissertation Presented

By

Sara Gozalo

Approved as to Style and content by:

Dr. Kenneth Rock, Chair of Committee

Dr. Raymond Welsh, Member of Committee

Dr. Joonsoo Kang, Member of Committee

Dr. Alan Rothman, Member of Committee

Dr. Leo Lefrancois, Member of Committee

Dr. Leslie J. Berg, Dissertation Mentor

Dr. Anthony Carruthers, Dean of the
Graduate School of Biomedical Sciences

Program in Immunology and Virology
May24th, 2004

Acknowledgements

I want to thank my family for all their constant support and love throughout the years. I would have never made it this far without them. My mom and dad, for their sacrifices, my sister Fuen for her strength, my brother Jaime for making me laugh, my grandparents for being amazing role models in my life, my aunt Tabel for her charm, and finally, my brother Juan for his help and encouragement, and for being there every time I needed him.

My mentor Leslie Berg has been a constant source of knowledge, support and understanding. She allowed me to be passionate about other aspects of my life, while encouraging me as a scientist. She pushed me to think on my own and gave me confidence in myself. For that, and more, I want to thank her.

The Berg lab, past and present. Every member of this group has been special for different reasons. Thanks to Morgan, Regina, Luana, Julie, Shane, Min, Heather, Katherine, Michael, Yoko, Martin, Andrea, Andy, and Zhongbin. I want to thank Luana, Shane, and especially Julie for their friendship and support from the first day of my graduate life. Andy, for his help in the lab and outside the lab, for his friendship, his honesty and for always making me smile. Morgan for being a role model both as a scientist and as a person, and for providing immeasurable help and support in and outside the lab. Andrea for being a sister to me, for her love, her honesty and her strength.

and outside the lab. Andrea for being a sister to me, for her love, her honesty and her strength.

A very special thanks to Mike Brehm for helping me with many experiments and computer problems, as well as for constantly discussing with me the meaning of our results.

I would like to thank all the members of my advisory thesis committee, Ray, Ken and Joonsoo, for their insights, their support, their help and their patience. I would also like to thank Dr. Rothman and Dr. Lefrancois for accepting to be a part of my defense committee. Also, many thanks to Dr. Cynthia Chambers, with whom I collaborated and who has been a great source of knowledge and enthusiasm.

I want to acknowledge the many mice that gave their lives for all the experiments described in this thesis.

ABSTRACT

T lymphocytes are essential components of the immune system and as such are continually regulated by a variety of factors. Every step of their development, survival and function is tightly monitored to ensure their ability to recognize most foreign agents and mount adaptive immune responses during pathogenic infections, while remaining tolerant to self-antigens. Among the many factors that participate in the regulation of T cell development and function are the cytokines. Cytokines that signal through the common gamma (γ_c) chain and the Janus kinase 3 (Jak3) include IL-2, -4, -7, -9, -15, and -21 and have been implicated in the regulation of every stage in the life of a T cell. Therefore, it is not surprising that mutations in the γ_c chain or Jak3 lead to a SCID condition in humans and mice. Specifically, Jak3-deficient mice are characterized by a reduction in thymic cellularity and dysregulated T cell homeostasis. They have an expansion of memory-like $CD4^+$ mature T cells and an almost complete absence of mature $CD8^+$ T cells. By investigating the TCR repertoire of $CD4^+$ T cells in the thymus and spleen of $Jak3^{-/-}$ mice, I deduced that the $CD4^+$ T cell activation and expansion is TCR-specific and takes place in the periphery of the mice. After crossing Jak3-deficient mice to Bcl-2 transgenic mice I showed that the developmental block observed in $Jak3^{-/-}$ mice could not be rescued by the anti-apoptotic factor, despite the fact that its expression did increase, slightly, the total numbers of developing thymocytes. The enforced expression of Bcl-2 was also not sufficient to revert the dysregulation of T cell homeostasis in $Jak3^{-/-}$ mice. Finally, in order to further understand the role played by γ_c cytokines during T cell function, I investigated the ability of mature $Jak3^{-/-}$ $CD8^+$ T cells

to become activated and differentiate into effector cells in response to a viral infection. My results indicate that $CD8^+$ T cells are activated and proliferate in response to a viral infection, but their survival, as well as their ability to proliferate and differentiate into effector cells are greatly impaired, resulting in the inability of Jak3-deficient mice to mount a protective response.

Table of Contents

List of Figures and Tables

Attributions

Chapter IV.

Julie Lucas assisted me with the stainings described in Figure 11.

Chapter V.

Dr. Andrew Miller assisted me with the in vitro stimulations and annexin V staining described in Figures 19 and 20. Dr. Morgan Wallace assisted me with the purification of dendritic cells described in Figure 19. Brian Sheridan assisted me with the plaque assays described in Table II.

CHAPTER I

INTRODUCTION

A. THE IMMUNE SYSTEM

The immune system is a fascinating and complicated network of many different cell types, organs and soluble factors that work in conjunction with each other to create a defense force, capable of attacking virtually every foreign antigen while ignoring self antigens. Among the most important components of this system are the lymphocytes, which, with the help of antigen presenting cells (APCs), are the conductors of adaptive immune responses against foreign pathogens. Under normal circumstances lymphocytes survive in a naïve state, only altered by infection with foreign pathogens. If lymphocytes encounter their specific antigen, they become activated and differentiate into effector cells capable of clearing the infecting pathogen. Once the antigen is cleared, the vast majority of activated lymphocytes die, leaving a small population of memory cells capable of recognizing and rapidly eliminating the antigen upon subsequent reinfection.

Given their importance for the proper function of the immune system, lymphocytes need to be tightly regulated throughout their lifetime. The consequences of a failure to properly regulate lymphocyte function are illustrated by the occurrence of autoimmune disease and allergy, where inappropriate lymphocyte responses are mounted against self-tissues or innocuous substances with debilitating effects. To minimize the chance of such failures, every step of lymphocyte development and function requires transition through a number of checkpoints dependent on a variety of cell surface receptors, intracellular signaling molecules and soluble factors. Developing lymphocytes are selected for their ability to recognize but tolerate self-proteins while maintaining the potential to respond to a broad repertoire of foreign antigens. Mature, antigen naïve

lymphocytes that successfully pass these developmental tests leave the primary lymphoid organs and enter the periphery where they circulate throughout the secondary lymphoid organs, awaiting encounter with their specific antigens. Their survival as naïve cells in the periphery, their ability to circulate throughout the body, and ultimately their activation following encounter with their cognate antigen are subject to further levels of regulation, mediated both by cell surface and soluble molecules.

Among the many factors that participate in the regulation of lymphocyte development and function are the cytokines. Cytokines are small soluble proteins secreted by one cell that can alter the behavior or properties of that same cell or another cell. Following receptor binding by a specific cytokine, signaling through the receptor ultimately induces new activities in the cell, such as proliferation, differentiation, or death. One family of cytokines that is of special importance for the development, survival and function of the immune system is the family that signals through the common gamma chain (γ_c) and the Janus kinase 3 (Jak3); IL-2, -4, -7, -9, -15, and -21. Although numerous studies have investigated the specific role played by the γ_c cytokines in the immune system, many questions remain unanswered. The overall goal of this dissertation is to further investigate the importance of γ_c cytokines for the immune system, specifically in T cell development, T cell homeostasis, and $CD8^+$ T cell function.

A.1 Lymphocyte Development

The development of lymphoid precursor cells into committed B and T cells proceeds through a series of stages, leading to the rearrangement of the antigen receptor

genes and expression of their protein products, the immunoglobins and the T cell receptors (TCRs). In addition to the production of antigen recognition structures, T and B cell precursors at each stage of development must also undergo changes in the expression of other cell-surface and intracellular proteins [1-4]. These changes in gene expression lead to the proliferation, differentiation and/or death of developing lymphocytes. To successfully complete development, B and T cell progenitors must pass through several checkpoints designed to ensure that cells that ultimately develop into mature functional lymphocytes can distinguish between foreign and self antigens, and mount appropriate immune responses against foreign pathogens while remaining tolerant to self proteins [3, 5, 6].

In mammals, T cell development takes place in the thymus, where bone marrow stem cells commit to the T cell lineage in response to signals from the thymic environment [7]. Thymocyte development can be simplified into three stages: the double negative stage (DN), characterized by the absence of the CD4 and CD8 coreceptors, the CD4 and CD8 double positive stage (DP), and the CD4 or CD8 single positive stage (SP) [8-12]. Cells become committed to the T cell lineage at the DN stage; this commitment is accompanied by proliferation and an upregulation of the survival factor Bcl-2 [13-15]. Although DN thymocytes are committed to the T cell lineage, at this stage they retain the potential to become either $\alpha\beta$ or $\gamma\delta$ T cells [16-18], and rearrangement of the γ and β chain TCR genes commences [19, 20]. $\gamma\delta$ T cells represent a T cell subset whose function is still not well understood, for the purpose of this literature review only $\alpha\beta$ T cell development will be discussed in detail. Cells that

proceed along the αβ TCR pathway first express their rearranged β chain at the cell surface in association with the non-rearranging pre-TCRα chain and the CD3/ζ proteins [21, 22]. The expression of the αβ pre-TCR is the first checkpoint of T cell development, and it is essential for further development. Signals received through the pre-TCR confirm that the thymocyte has produced a functional TCR β-chain and initiate the rearrangement of the endogenous α locus, leading to the surface expression on the αβ TCR assembled with CD3/ζ proteins [3, 23]. Pre-TCR signals also trigger the expression of the CD4 and CD8 coreceptors, marking the transition to the DP stage [3, 12, 24].

Although DP thymocytes represent the largest lymphoid compartment in the thymus, most of these cells are destined to die through the processes of positive and negative selection, which together act to generate a self-restricted T cell population with a sufficiently diverse repertoire of TCRs to mount appropriate immune responses against a broad spectrum of foreign pathogens, while eliminating cells that might respond inappropriately to self tissues [12, 25].

The TCR specifically recognizes antigens that have been processed into peptides, presented by major histocompatibility complex (MHC) molecules on the surface of APCs [26, 27]. T cells expressing TCRs that recognize foreign peptides presented by self-MHC form the basis of a functional adaptive immune system. Meanwhile the expression of TCRs that recognize self peptides is not beneficial to the host and can potentially lead to autoimmunity [28]. Therefore, the ultimate goal of thymocyte development is to select T cells expressing a TCR with high avidity for non-self peptides presented by self-MHC molecules while eliminating potential self-reactive T cells [29, 30]. The DP stage of

thymocyte development is where self-tolerance is imposed on the developing T cells and as such, represents a critical checkpoint in the generation of a functional adaptive immune system. Most of the DP thymocytes that are generated express TCRs that interact poorly with the self-peptide-self-MHC complexes presented in the thymus, these cells fail to recognize self MHC with sufficient avidity to receive the necessary survival signals and will instead die by neglect [24, 31]. Others express TCRs that interact with self-peptide-self-MHC complexes in the thymus with too high an affinity; these lymphocytes could cause autoimmunity if they were to enter the periphery and are therefore killed via apoptotic mechanisms during negative selection [12]. The thymocytes that express TCRs that have an intermediate affinity for self-MHC (a degree of affinity that lies between that which results in neglect and that which results in apoptosis) are positively selected, upregulating the anti-apoptotic protein Bcl-2 and undergoing the process of T cell lineage commitment whereby expression of either CD4 or CD8 is downregulated, resulting in a progression to either the CD4 or CD8 single positive stage of T cell development [32-39]. This differentiation is also accompanied by other genetic events that will dictate whether the mature T cell can become a helper, or cytotoxic effector cell, upon stimulation [12, 40, 41].

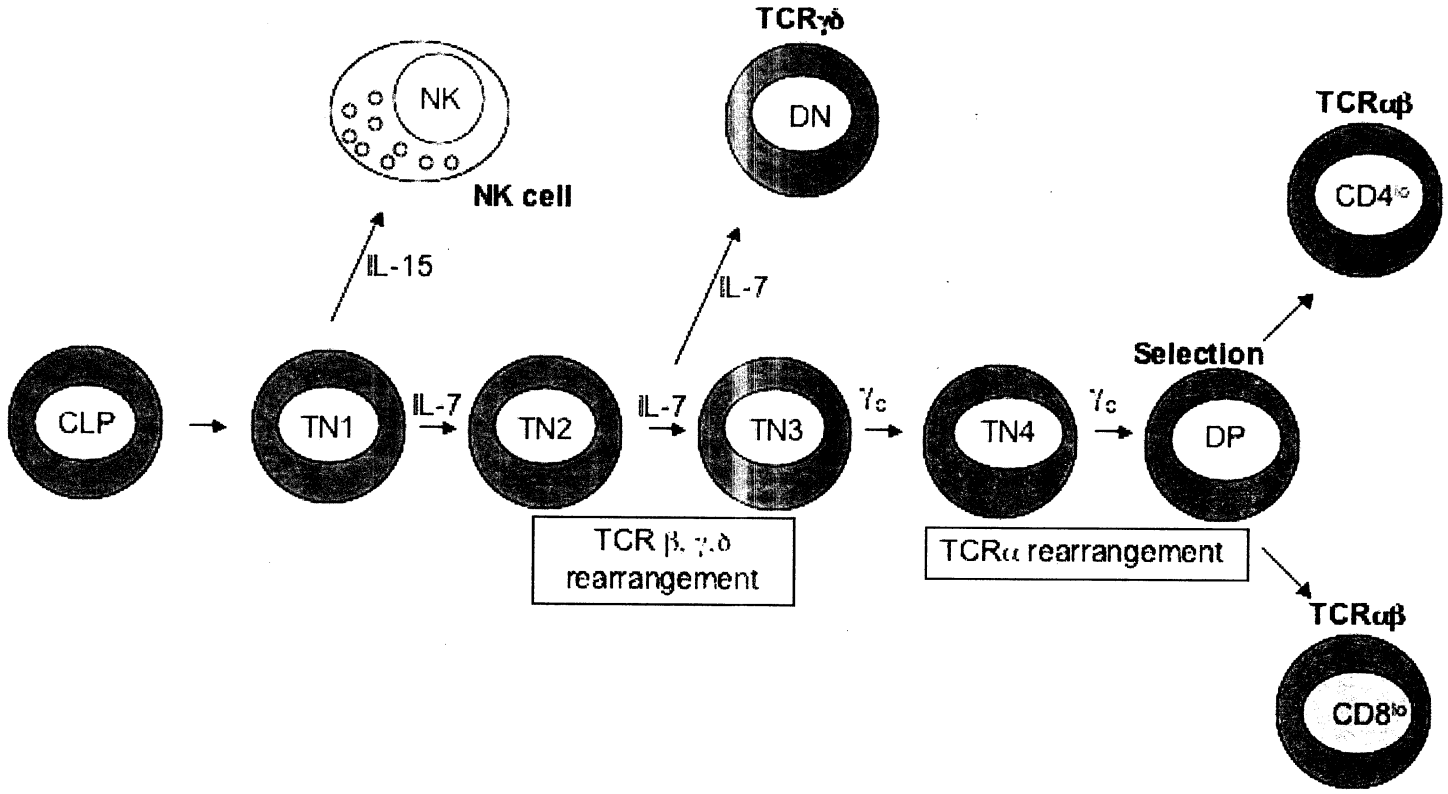
Every stage of thymocyte development, including the rearrangement of the TCR chains and the survival and differentiation of thymocytes into mature T cells, is tightly regulated by cell differentiation programs, cell-cell interactions, as well as soluble factors [42-44]. For example, Fine and Kruisbeek [45] demonstrated that interactions between the LFA-1 integrin (on thymocytes) and ICAM-1 (on thymic stromal cells or thymocytes)

are important for the maturation of DN thymocytes to the DP stage. As mentioned before, interactions between the TCR on thymocytes and MHC-peptide complexes on molecules of the thymic stroma are essential during thymic selection, and only cells bearing TCRs with the potential to recognize foreign peptides in the context of self-MHC will mature into $CD4^+$ or $CD8^+$ SP T cells [46].

Among the soluble factors that contribute to the process of T cell development, cytokines that signal through the γ_c chain [11, 43] are especially important. For instance, the γ_c cytokine IL-7 is required at different stages of T cell development, supporting early T cell progenitor survival, initiating TCR-γ chain rearrangement, and promoting $CD8^+$ thymocyte positive selection [44, 47, 48]. Another γ_c cytokine, IL-15, is essential for the development of NK cells in the thymus. The absence of this cytokine causes a block in NK cell development, which leads to the absence of mature NK cells [49, 50] (Fig.1). The specific role played by the γ_c chain cytokines during T cell development will be further described in section C.1 of the introduction.

Fig.1 γ_c cytokines play an important role in lymphocyte development in the thymus (adapted from [51])

Bone marrow stem cells enter the thymus where they commit to the T or NK cell lineage. NK cells depend on IL-15 mediated signals to fully develop into mature cells. At the $CD3^-$ $CD4^-$ $CD8^-$ TN stage of development, TCR gene rearrangements begin, and committed T cells develop into the αβ or γδ T cell lineage. Signals mediated through the IL-7R are required for the rearrangement of the γ chain, and thus for the development of γδ T cells. Throughout different stages of αβ T cell development, signals mediated by the γ_c chain are required to maintain the survival and proliferation of developing thymocytes. Nevertheless, αβ T cells develop in the absence of these signals, though the total numbers of αβ thymocytes and SP $CD4^+$ and $CD8^+$ immature T cells are greatly reduced.


TCRγδ
NK
DN
TCRαβ
CD4lo
NK cell
IL-15
IL-7
IL-7
Selection
CLP
TN1
IL-7
TN2
IL-7
TN3
γc
TN4
γc
DP
TCR β, γ, δ rearrangement
TCRα rearrangement
TCRαβ
CD8lo

A.2 T cell homeostasis

On leaving the thymus, newly matured naïve $CD4^+$ and $CD8^+$ T cells enter the periphery where they circulate through the secondary lymphoid organs, awaiting recognition of invading pathogens [52]. Following encounter with their specific antigen T cells become activated, undergoing rapid proliferation and differentiation into mature effector cells, and migrate out of the secondary lymphoid organs to the tissues where they eliminate the foreign antigen [52, 53]. Naïve T cells have a limited lifespan, and if they do not encounter their specific antigen after a defined period of time, will die [54-56]. This limitation of their lifespan is important in the maintenance of constant numbers of lymphocytes in the face of continuous production of naïve T cells by the thymus, a process termed T cell homeostasis. Both T and B lymphocytes are continually produced throughout life, either in primary lymphoid organs or by peripheral cell division, but the total lymphocyte cell number remains constant [57-59]. During an immune response the number of specific T cell clones is expanded, but this expansion is immediately followed by a contraction, achieved via the apoptosis of activated cells [60-63]. A successful immune response is also accompanied by the creation of a new small memory T cell population [64]. Therefore, once the infection is cleared the total number of peripheral T cells returns to homeostatic equilibrium. Maintenance of constant numbers and proportions of lymphocytes in both the naïve and antigen-experienced memory compartments is essential to sustain immunocompetence [57].

There is good evidence that lymphocyte numbers are tightly regulated in mice and humans [7]. T cell numbers in the spleen and lymph nodes of different laboratory strains

of mice, as well as wild mice are very consistent at any given age [65]. Transgenic mice that express rearranged TCRs do not have more T cells than normal, even though every thymocyte could potentially mature into a T cell in the presence of the appropriate MHC [66]. The ratio of $CD4^+$ to $CD8^+$ T cells is also very consistent in any given mouse strain [66, 67]. This ratio may change during the course of an immune response, for example during a viral infection, but it returns to equilibrium once the pathogen is cleared. Additional confirmation of T cell homeostasis is manifested when normal mature T cells are transferred into lymphopenic mice, which exhibit an absence of normal numbers of peripheral T cells [66, 68, 69]. The transferred T cells divide, increasing the total T cell numbers and returning to normal WT levels [68-70]. This phenomenon, known as homeostatic proliferation, does not take place when cells are transferred into mice with normal T cell numbers. Both $CD4^+$ and $CD8^+$ T cells display this phenomenon.

The precise mechanisms that regulate peripheral lymphocyte homeostasis, as well as homeostatic proliferation are not fully understood. However, factors such as cytokines and cell surface molecules that are known to play vital roles in T cell survival also seem to be essential for homeostasis and homeostatic proliferation [57, 58, 68, 70-75]. Under normal circumstances naïve T cells do not divide in mature animals, but they do require exogenous factors to stay alive [66, 76]. During their migration through the lymphoid organs, T cells are exposed to a multitude of soluble and cell-associated proteins that likely play a role in their survival and homeostasis. The different T lymphocyte populations (i.e. naïve vs. memory, $CD4^+$ vs. $CD8^+$) depend on different mechanisms for survival, and/or homeostatic proliferation. Several groups have demonstrated that naïve

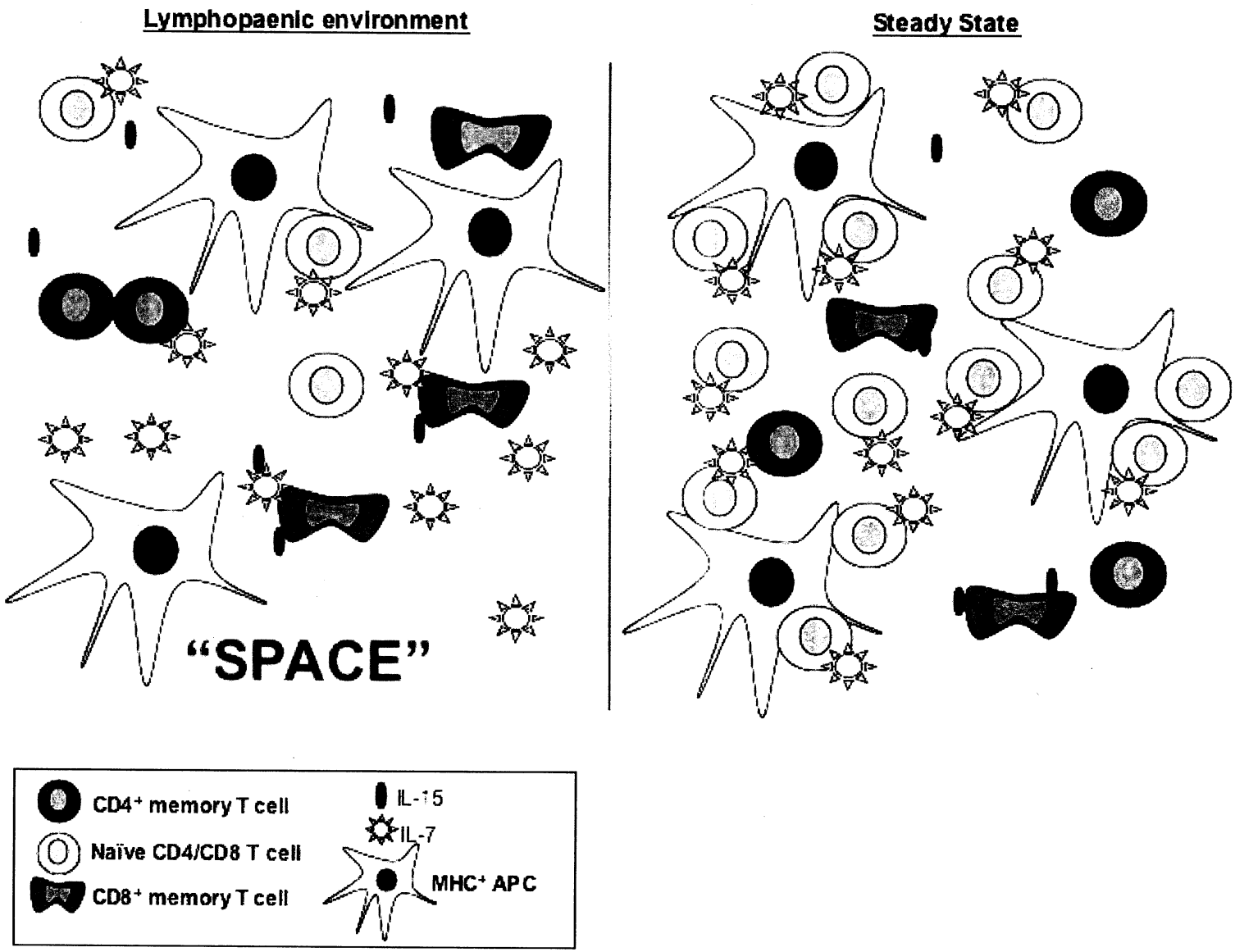
T cells (both $CD4^+$ and $CD8^+$) require TCR contact with MHC class II and class I molecules, respectively, expressing a diversity of self-peptides to survive [69, 77, 78]. Thus, naïve T cells transferred into MHC-deficient mice die rapidly, whereas they survive for long periods of time when transferred into mice expressing the appropriate MHC molecules [58, 71, 72]. Memory T cells, on the other hand, are thought to survive for long periods in the absence of MHC molecules. This has been demonstrated by experiments in which memory $CD4^+$ and $CD8^+$ T cells survive almost indefinitely upon transfer into $MHC^{-/-}$ hosts [79, 80].

In contrast to their differing dependence on the presence of MHC, both naïve $CD4^+$ and $CD8^+$, as well as memory $CD8^+$ T cells are dependent on specific cytokines to survive and homeostatically proliferate. Of particular importance are the γ_c cytokines, and defects in one or more specific members of this family may lead to the dysregulation of naïve T cell survival and T cell homeostasis. Further details about the specific roles played by γ_c cytokines during T cell homeostasis and homeostatic proliferation will be described in section C.2.

Two hypotheses that have been put forward as mechanisms underlying lymphocyte homeostasis and homeostatic proliferation are 1) competition between T cells for access to the finite number of MHC molecules or 2) the limited availability of cytokines. As mentioned above, homeostatic proliferation only occurs when total T cell numbers are low [81]. The role of cytokines in homeostatic proliferation is supported by a growing body of evidence [57, 73] (Fig.2).

Fig.2 The Space model of T cell homeostasis (adapted from [81])

IL-7 and IL-15 are essential for the survival and homeostatic proliferation of T cells. MHC molecules expressed on APCs are necessary for the survival of naïve T cells. Therefore, in a lymphopenic environment in which IL-7 and IL-15, as well as MHC molecules are more readily accessible, a limited population of mature T cells may be induced to homeostatically proliferate, leading to increased numbers of peripheral lymphocytes. In a normal steady-state environment, MHC molecules, as well as cytokines, are engaged by the presence of a substantial number of mature T cells. Transferring a population of mature T cells into this environment does not lead to their proliferation.


Lymphopaenic environment
Steady State
"SPACE"
CD4+ memory T cell
Naïve CD4/CD8 T cell
CD8+ memory T cell
IL-15
IL-7
MHC+ APC

Several reports have revealed that overexpression of IL-7 increases the size of the T cell pool and supports homeostatic proliferation [73, 82, 83]. Also in support of this hypothesis is the observation that there is an inverse correlation between the number of T cells and the amount of IL-7 in the serum of AIDS patients [84, 85]. This observation would suggest that either T cells take up free IL-7 or that stromal cells produce more IL-7 in response to a decline in the normal numbers of T cells. In addition, the observation that $CD4^+$ T cells inhibit proliferation of $CD8^+$ T cells in irradiated MHC class-II deficient hosts [55, 86] provides evidence against the availability of MHC/peptide complexes on APCs causing homeostatic proliferation. However, it is possible that homeostatic proliferation occurs in the absence of competition for factors in T cell areas, such as cytokines, chemokines, or other molecules on APCs.

During an immune response the total number of lymphocytes is significantly increased and the CD4:CD8 ratio may also be altered due to the clonal expansion of the specific responding T cells. However, T cell homeostasis is immediately restored following the clearance of the pathogen. This restoration is mediated by apoptosis through activation-induced cell death [87]. Therefore, the maintenance of T cell homeostasis is also dependent on the proper regulation of T cell death [61, 62, 66]. The mechanisms that regulate this process are discussed in the following section.

A.3 T cell-mediated immune responses

Following T cell development in the thymus, mature naïve T cells enter the periphery and circulate between the blood and the T-cell rich regions of secondary

lymphoid organs until they encounter their specific antigen [54-56]. During an infection, antigenic peptides are presented to T cells in the context of self MHC molecules by resident lymphoid APCs that acquire antigen brought to the lymphoid tissue and recent APC migrants from the site of infection [52, 88-90]. T cell stimulation requires two separate signals [91-94]. Signal one is mediated by TCR recognition of its specific self-MHC-peptide complex on an APC. Signal two, known as the costimulatory signal, is mediated by the binding of one or more costimulatory/adhesion molecules on T cells to their ligands on APCs [92, 95, 96]. One of the most important and well defined T cell costimulation pathways is between CD28 on T cells and B7-1/B7-2 on APCs [91, 92, 97]. Induction of inflammation during an infection causes a dramatic upregulation of B7-1 and B7-2 on the APCs. This allows APCs to costimulate T cells much more effectively [97]. Other costimulatory molecules have also been described in recent years, for example PD-1, ICOS and OX40, but their precise contribution to T cell stimulation remains to be clearly determined [98, 99]. CTLA-4, a homologue of CD28 expressed by T cells, also binds to the B7 molecules, but acts as a negative regulator of T cell activation [100, 101].

T cell activation is followed by a clonal expansion and a genetic reprogramming that allows T cells to become effector cells [52, 97, 102]. This phenomenon also leads to a change in expression of certain cell surface molecules, such as CD44 and CD62L in mice, which are characteristic markers used to differentiate between naïve, activated and memory T cell subsets [103]. Naïve T cells express low levels of CD44 and high levels of CD62L, while activated T cells express high levels of CD44 and low levels of CD62L.

Memory cells are also high in CD44 and they may or may not upregulate the expression of CD62L [103-105]. These changes allow activated T cells to leave the secondary lymphoid organs and migrate to sites of infection. Upon differentiation into effector cells, activated T cells also become capable of producing cytokines and other soluble factors that will mediate the clearance of the infecting pathogen [104, 106-108].

In addition to the two signaling pathways initiated by the TCR and CD28, the production of cytokines and signals received through cytokine receptors are essential for the production and maintenance of effective immune responses [102, 109-111]. Cytokine signaling is important for the initial steps of T cell activation, as well as the development and maintenance of a proper, strong immune response. $CD4^+$ and $CD8^+$ T cells differ in their specific roles during immune responses. Depending on the nature of the infection, $CD4^+$ T cells may differentiate into either Th1 or Th2 cells, characterized by the secretion of specific cytokines. These cytokines will aid and modulate the type of effector response [112, 113]. $CD8^+$ T cells, on the other hand, generally differentiate into cytotoxic T lymphocytes (CTLs) that help clear pathogens by killing infected cells. To accomplish this, $CD8^+$ T cells can express high levels of cytokines, such as IFN-γ and TNF-α, and upregulate the expression of other proteins, such as FasL, perforin and granzymes that mediate lysis of cells expressing the specific antigen recognized by that T cell [114-116]. The activation and differentiation of both $CD4^+$ and $CD8^+$ T cells determine the resistance or susceptibility of the host to foreign pathogens.

Once the infecting pathogen has been cleared, activated T cell expansion is quickly followed by a clonal contraction, leaving only a small memory T cell population

that provides lasting immunity against a secondary attack by the same pathogen [52, 97, 102]. Activated T cells become sensitive to death via activation-induced cell death (AICD), following ligation of the TCR with its specific peptide, when the cells are already in cell cycle [87, 117, 118]. Since apoptosis of activated T cells is extremely important for restoring T cell homeostasis after an immune response, there are several mechanisms that can mediate this process. AICD can be mediated by Fas/FasL interactions, in which expression of FasL on activated T cells induces apoptosis of other T cells expressing Fas [119, 120]. Signaling through Fas leads to the activation of specific caspases that mediate cellular apoptosis [121, 122]. Several studies have shown that the γ_c cytokine IL-2 plays a critical role in the induction of AICD [123-125]. IL-2 is involved in regulating T cell immune responses at multiple levels including activation, proliferation and differentiation of T cells [126, 127]; however, IL-2 deficient mice do not present an immunodeficient phenotype, but suffer from T cell lymphoproliferation [123, 128, 129]. These observations would suggest that perhaps the most important role of IL-2 is during the process of T cell death. It has been demonstrated that IL-2 induces AICD, mainly by the upregulation of FasL expression; thus T cells deficient in IL-2-mediated signals fail to upregulate FasL and may be resistant to Fas-induced apoptosis [63, 130, 131]. However, a new school of thought proposes that the lymphoproliferation observed in IL-2-deficient mice is mostly due to the absence of IL-2-dependent $CD25^+$ $CD4^+$ regulatory T cells [132, 133].

Another cytokine produced by activated T cells is TNF-α, which induces apoptosis of T cells through the activation of caspases, following binding to its cell

surface receptor [134]. Recent reports have brought attention to members of the Bcl-2 family as mediators of T cell death [135]. Hildeman et al investigated the importance of one member of this family, Bim, during T cell activation induced cell death. They discovered that overexpression of Bim, as well as Bad (also a member of the Bcl-2 family), resulted in the accelerated death of $CD4^+$ and $CD8^+$ T cells following SEB-mediated activation. Also, when Bim-deficient mice were injected with SEB, about 80% of the SEB-reactive T cells *in vivo* were prevented from undergoing AICD. Given the discrepancies between these results, which suggest that Fas is dispensable for SEB-induced AICD and previous results in which Fas appeared to play an essential role, Hildeman et al. suggested that the difference may be a result of the experimental design. In studies where animals were injected with superantigen repeatedly, Fas seemed to play a important role in AICD, whereas in studies in which animals were injected with a single does of the superantigen (as was the case in this particular study) AICD appeared to be Fas-independent [135]. Another mechanism whereby cytokines contribute to the contraction of T cell numbers in the terminal stages of an immune response is due to the withdrawal of growth factors, known as cytokine deprivation death (CDD) [136]. CDD is mainly controlled by members of the Bcl-2 family. Following T cell activation, levels of Bcl-2 are downregulated, shifting the ratio of anti-apoptotic to pro-apoptotic factors and leading to the death of unwanted T cells after an immune response [87].

In summary, to generate a successful immune response that will clear an infection; mature naïve T cells must recognize specific antigen in the form of an MHC/peptide complex, in the context of costimulatory molecules such as B7-1 and B7-2

expressed on the surface of APCs. These activating signals must be accompanied by the production of specific cytokines, such as IL-2, and by subsequent signals through cytokine receptors. Together, these signals regulate the duration and type of immune response, triggering the differentiation of $CD4^+$ T cells into Th1 or Th2 cells that secrete different cytokines, while $CD8^+$ T cells will develop into CTLs producing cytokines, such as IFN-γ and TNF-α, and the effector molecules FasL, perforin, and granzymes that will mediate the killing of infected cells. Finally, a successful immune response will also generate a population of memory T cells to provide life-long immunity against that specific antigen [137].

Many aspects of the process of lymphocyte development, homeostasis and function are regulated by cytokines, all of which signal through a related set of receptors [137]. The receptors are composed of one to three chains and are associated with one or more members of the Janus family of protein kinases. This dissertation investigates the role played by one of its members, Jak3, in the proper function of T cell immunity.

B. JAKS AND STATs

The Janus family of tyrosine kinases is composed of four non-receptor tyrosine kinases, Jak1, Jak2, Jak3 and Tyk2, that associate with specific chains of different cell surface cytokine and growth factor receptors [138-140] (Table. I).

Table I. Cytokine receptors and their specific Jak/STAT mediators

CYTOKINES	JAKs/STATs
Type II cytokines	
IFNαβ	Jak1, Tyk2/STAT1, 2, 4(human)
IFNγ	Jak1, Jak2/STAT1
IL-10	Jak1, Tyk2/STAT3
Cytokines whose receptors share γ_c	
IL-2, -4, -7, -9, -15, -21	Jak1, Jak3/STAT5a, b, 6
IL-13 (uses IL4Rα not γc)	Jak1, Jak2, Tyk2
Cytokines whose receptors share β_c	
IL-13, -5, GM-CSF	Jak2/STAT5a, b
Cytokines whose receptors share gp130	
IL-6, IL-11, OSM, CNTF, LIF, CT-1	Jak1, Jak2, Tyk2
IL-12 (uses a gp130-related receptor)	Jak2, Tyk2/STAT4
Cytokines with homodimer receptors	
Growth hormone, Prolactin, EPO, TPO	Jak2/STAT5a, b

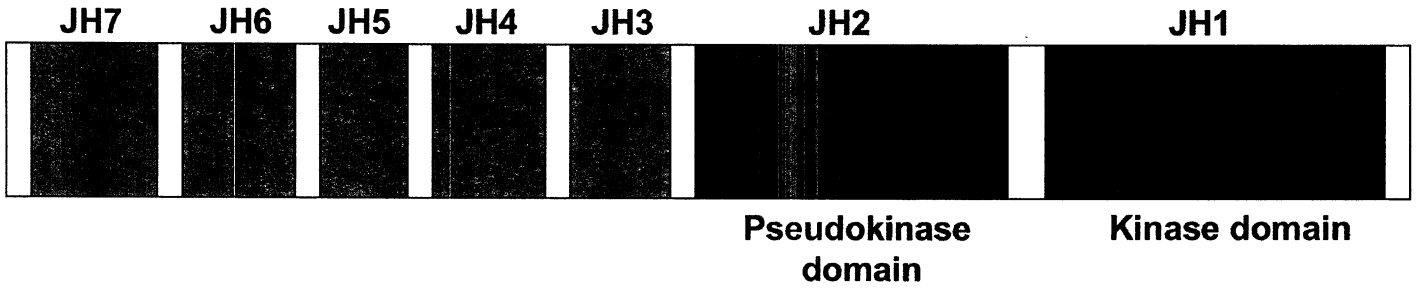
Jaks are large kinases with molecular weights ranging from 120 to 140 KDa., and are composed of seven regions of homology termed JH1 through JH7 [138] (Fig.3 A). The name Janus comes from the mythological Roman god who had two faces, and refers to the observation that Jaks have two kinase-like domains. The JH1 domain is a functional catalytic domain whereas the JH2 domain is a pseudokinase domain whose function is currently debated. Some experiments have suggested that the pseudokinase domain inhibits Jak activity, while others have suggested that it is actually required for kinase activity [141-143]. Additional data have indicated that this domain may constitute a potential docking site for a family of transcription factors known as signal transducers and activators of transcription, or STAT proteins [142]. The exact function of the other five blocks of homology (JH3 through JH7) remains unclear, however, the N-terminal region has been shown to bind cytokine receptors [144-147]. Jaks are essential for cytokine receptor signaling, as was demonstrated using Jak mutant cell lines that failed to respond to interferons (IFNs), unless restored with genes encoding different Jaks [148, 149]. Furthermore, experiments conducted with cytokine receptor-mutant cells that fail to bind Jaks, and experiments with cells expressing a dominant negative Jak construct also confirmed the essential role of these kinases in cytokine signaling [150-153].

The STAT family of transcription factors is composed of seven members, STAT1 through STAT4, STAT5a and STAT5b, and STAT6 [154]. All STAT proteins share functional domains, including a Src-homology 2 (SH2) domain that recognizes phosphorylated tyrosines on receptor subunits (Fig.3 B).

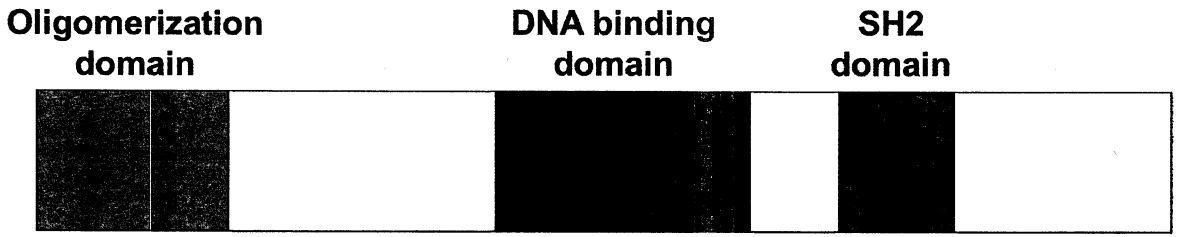
Fig.3 Structure of JAK and STAT proteins

Jak proteins are composed of seven regions of homology. The JH1 is a functional catalytic domain, while the JH2 domain is structurally similar but functionally inactive. The exact function of the other domains remains unclear. All STAT proteins share functional domains, including a receptor binding SH2 domain, a STAT DNA domain, and an oligomerization domain. A conserved tyrosine in the oligomerization domain becomes phosphorylated by activated Jaks, allowing STAT proteins to then form dimers, through the interaction of the SH2 domain of each STAT and the phosphorylated tyrosine on the other.

A) Structure of Jaks



B) Structure of STATs

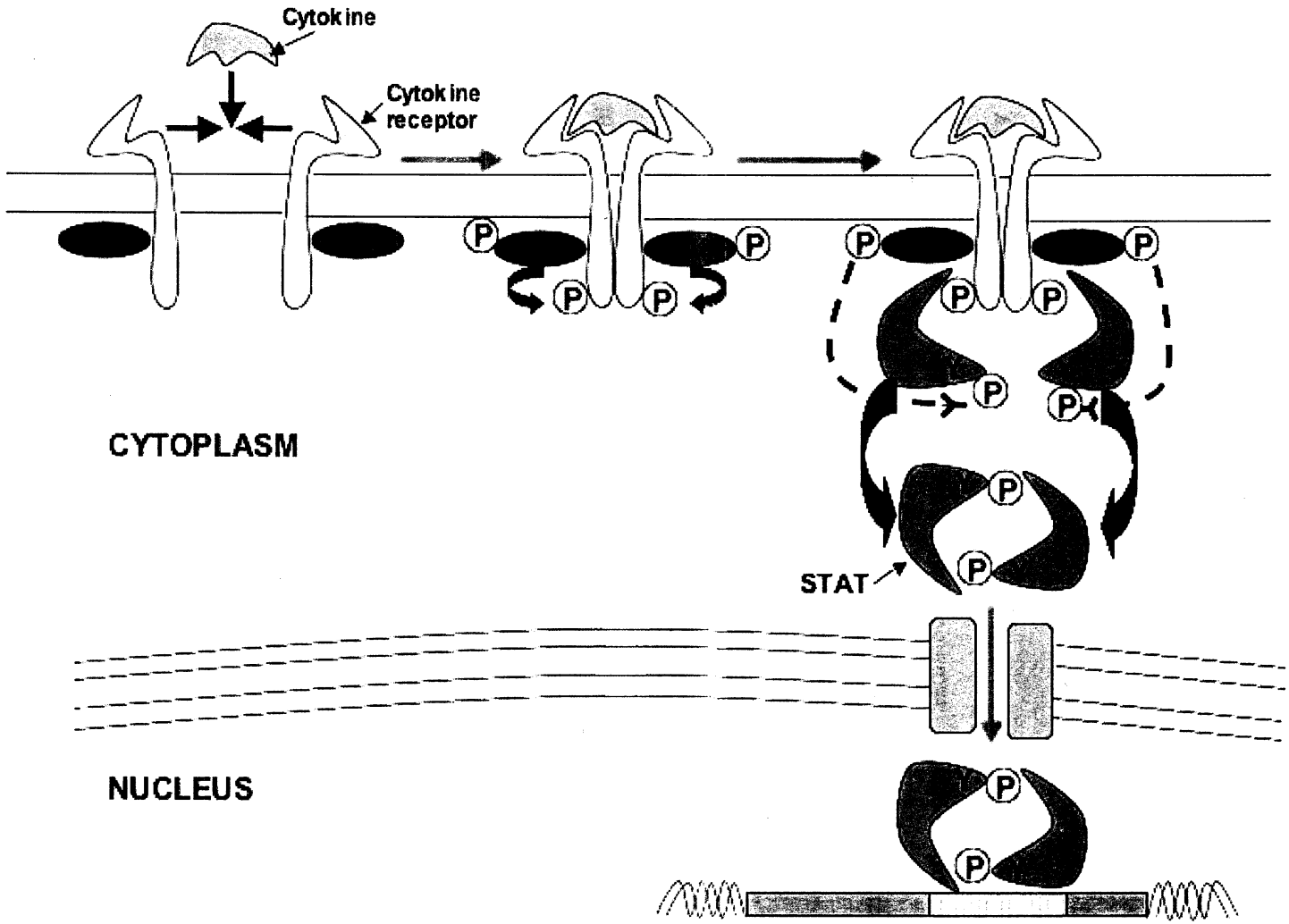


Differences in the SH2 domain of the STAT members determine their cytokine receptor binding selectivity, because, in addition to recognizing the phosphorylated tyrosines, these domains specifically recognize surrounding residues [140, 155]. Although a STAT DNA-binding motif has been defined (TTGNNCAA), the precise composition and spacing of the sequence determines which of the seven members preferentially binds [137, 156].

Cytokine binding to its receptor leads to the homo- or hetero-dimerization of the receptor's subunits, bringing associated Jaks into close proximity, allowing the Jaks to auto- and trans-phosphorylate each other, resulting in their full activation [139, 154, 157]. Once activated, Jaks in turn phosphorylate the cytokine receptor chains, generating docking sites for the STATs SH2 domains [158, 159]. Immediately after STAT binding, a conserved tyrosine is phosphorylated by the activated Jaks, allowing STAT proteins to form dimers through interactions between the SH2 domain of one STAT and the phosphorylated tyrosine of another [160, 161]. Recent reports from Kenneth Murphy's lab have shown that STAT dimers are formed in vivo independent of cytokine receptor-mediated signals and that these dimers are required for the receptor-driven phosphorylation and activation of STATs [162]. The investigators have suggested that this dimerization may facilitate a rapid formation of activate dimers following activation. In addition, the N-terminal domain of the STAT protein is involved in oligomerization of STAT dimers to form tetramers, or even highly order oligomers, which may be important for transcriptional activation [163]. Once oligomerized, STATs translocate into the nucleus where they bind their consensus DNA motifs on target genes (Fig.4).

Fig.4 The Jak-STAT-mediated cytokine signaling pathway (adapted from [164])

Upon cytokine binding, the receptor subunits are brought in close proximity to each other allowing for the auto- and trans-phosphorylation of receptor-associated Jak proteins. Activated Jaks phosphorylate specific sites on the receptor subunits, creating docking sites for the SH2 domains of STAT molecules. STATs bind to the receptor chains and are in turn phosphorylated by Jaks. Once phosphorylated, STATs homo- and hetereo-dimerize and translocate to the nucleus where they bind to promoter sites of genes required for cell cycle progression and differentiation.


Cytokine
Cytokine receptor
P
CYTOPLASM
STAT
NUCLEUS

The activation of STATs is one of the major functions of Jaks, but they also play other roles in signaling. For example, Jak proteins have been implicated in the activation of the extracellular signal-regulated kinase subfamily of the mitogen-activated protein kinases (ERK/MAPK) pathway following cytokine binding [164, 165]. This activation is abrogated if mutations are introduced in the Jak-binding domain in several cytokine receptors, suggesting a link for the activation of the ERK/MAPK pathway by cytokines [166]. Therefore Jak proteins seem to function upstream of two signaling pathways, the STAT pathway and the ERK/MAPK pathway, which most likely results in the enhancement of the transcription of genes regulated by STATs and the ERK/MAPK-activated transcription factors [164]. Several studies have also demonstrated that Jak proteins may modulate PI-3 kinase function. For example, activation of PI-3K following GM-CSF binding to its receptor is abrogated by treating the responding cells with AG-490, a Jak2 inhibitor [167, 168].

Negative regulation of the Jak-STAT pathway is mediated by several different mechanisms. These include the endosomal degradation of Jak/receptor complexes through receptor-mediated endocytosis, the dominant-negative effects of naturally occurring STAT proteins, regulation by the CIS/SOCS/SSI family and by tyrosine phosphatases [155, 169-173]. CIS family members mediate their negative effects by different mechanisms, which include direct binding to the catalytic domain of Jaks, and binding to the cytokine receptor, where they may prevent stimulatory signaling coupled to phosphotyrosine motifs on the receptor [155, 174, 175]. Additionally, since CIS proteins have short half-lives, they may target activated receptors for degradation [175].

Tyrosine phosphatases containing tandem SH2 domains bind either the cytokine receptor or the Jaks, leading to their dephosphorylation, leading to reduced activation of the Jak-STAT pathway [176].

C. JAK 3

Jak3 is unique among the Janus kinases because it is preferentially expressed in hematopoietic cells, where it constitutively binds to the cytokine receptor γ_c chain [150, 151, 177, 178]. The group of cytokines that bind to this receptor (IL-2, -4, -7, -9, -15, and -21) plays important roles in different aspects of lymphocyte development, homeostasis and function. In 1995, several groups observed that the absence of Jak3 in mice led to a phenotype that was virtually identical to the phenotype of mice lacking the γ_c chain [179, 180]. Their importance is illustrated by the discovery that defects in γ_c chain- or Jak3-mediated signaling lead to severe combined immunodeficiency (SCID) in both humans and mice [179-182]. Interestingly, the absence of γ_c/Jak3-mediated signals leads to contrasting defects between mice and humans, revealing differences in cytokine requirements during lymphocyte development in these two species [183, 184]. By examining the phenotype of mice deficient in single γ_c-cytokine or γ_c-cytokine receptor subunits, it has been possible to elucidate which cytokines are important for different aspects of lymphocyte development, homeostasis, and function, as is discussed in the following sections.

C.1 Jak3/γ_c signals in lymphocyte development

As mentioned above, signals mediated by the γ_c chain cytokines are extremely important during lymphocyte development. B cell development in γ_c- and Jak3-deficient mice is arrested at the pro-B cell stage, leading to an absence of mature B cells [179, 180]. This defect is also present in mice that lack IL-7-mediated signals, indicating the importance of IL-7 for murine B cell development [185, 186]. Interestingly, humans who lack γ_c or Jak3 signals appear to have normal B cell development, suggesting that IL-7 is not essential and that a γ_c-independent cytokine must be responsible for the production of mature human B cells [183, 184]. In mice, IL-7 regulates transcription and rearrangement of the IgH genes, and signaling through its receptor induces expression of anti-apoptotic factors, such as Bcl-2 [44, 187].

IL-7-mediated signals are critical for T cell development. The thymi of γ_c and Jak3-deficient mice are extremely reduced in cellularity, with a size about one tenth of that of a normal thymus. The rate of cell division in double negative and double positive stages is also diminished [17]. Transition between the earliest stage of T cell lineage, the pro-T cell stage, to the pre-T cell stage is blocked in the absence of IL-7 (Fig.1) [186, 188]. γ_c-mediated signals, especially IL-7-mediated signals, induce expression of Bcl-2, providing anti-apoptotic signals during development [17]. The normal upregulation of Bcl-2 expression from DN $CD44^+CD25^-$ to DN $CD44^+CD25^+$ and from DP TCR^{lo} to DP TCR^{int} is impaired in γ_c-deficient mice [16, 187]. Additionally, DN and $CD4^+$ or $CD8^+$ SP thymocytes have a higher frequency of apoptosis in γ_c knock-out mice compared to WT mice, correlating with reduced levels of Bcl-2 expression by these cells [49].

Despite the reduced numbers of developing thymocytes, the individual stages of αβ T cell development in γ_c-, Jak3-, and IL-7-deficient mice seems to take place normally. The proportions of maturing αβ thymocyte subsets are comparable to WT mice, suggesting that Jak3/γ_c-mediated signals are important but not essential for the development of αβ thymocytes in mice [43, 179, 189]. Deficiencies in these signals in humans cause a complete arrest in αβ thymocyte development, leading to an absence of mature αβ T cells [184]. Several studies, using γ_c-deficient mice have suggested that γ_c/Jak3-mediated signals are not required to negatively select thymocytes expressing self-reactive TCRs [11, 16]. When γ_c-deficient mice were crossed to the H-Y TCR transgenic mice, in which all the T cells are specific for the male-specific antigen HY, thymocytes were negatively selected in male mice [11]. Additional experiments, using superantigen models to study negative selection, also revealed that thymocytes can be negatively selected in the absence of γ_c signals [16].

The γ_c cytokines IL-2 and IL-15 may also play a role in the survival of $CD8^+$ SP thymocytes during development. Analysis of mice deficient in SOCS-1, which is expressed by all thymocytes during the different developmental stages, and functions as a negative regulator of cytokine receptor signaling, revealed that IL-2 and IL-15 may influence CD8 thymocyte maturation by inducing the expression of Bcl-xL, and cell proliferation [190].

In contrast to αβ T cell development, mice deficient in γ_c chain, Jak3, or IL-7R, completely lack γδ T cells, and TCRγ chain rearrangement is undetected in adult thymi [191, 192]. The number of γδ T cells is still severely reduced in γ_c-deficient mice

expressing a functionally rearranged TCR Vγ gene. Even after taking into account the lower levels of thymocyte precursors present in the thymus of $\gamma c^{-/-}$ mice, γδ T cells were still reduced eightfold in γ_c^- Tg mice relative to γ_c^+ Tg mice [191]. These results suggest that the γ_c cytokine receptor provides essential survival signals, in addition to the signals required for the rearrangement of the TCRγ locus. However, high copy transgenic expression of the γ chain in IL-7R knock-out mice led to the production of normal numbers of γδ T cells, suggesting that signals through the IL-7 receptor are essential for the rearrangement of the γ chain but not for the survival of γδ T cells [48]. The discrepancy in these results may reflect the importance of another γ_c cytokine during γδ T cell development, or it may be a consequence of the level of expression of the transgenic gene, since in a low copy γ transgene failed to rescue development of these cells in the $IL\text{-}7R^{-/-}$ system [48].

The development of NK cells in γ_c/Jak3 knock-out mice is also impaired at very early stages, also leading to a complete absence of these cells in the periphery. NK cell development is blocked in mice deficient in IL-15, but not in mice deficient in IL-7-mediated signals. Additionally, IL-15 has been shown to induce NK cell differentiation from bone marrow derived hematopoietic progenitor cells [193, 194], suggesting that NK cell development is critically dependent on IL-15 signals.

C.2 Jak3/γ_c signals in T cell homeostasis

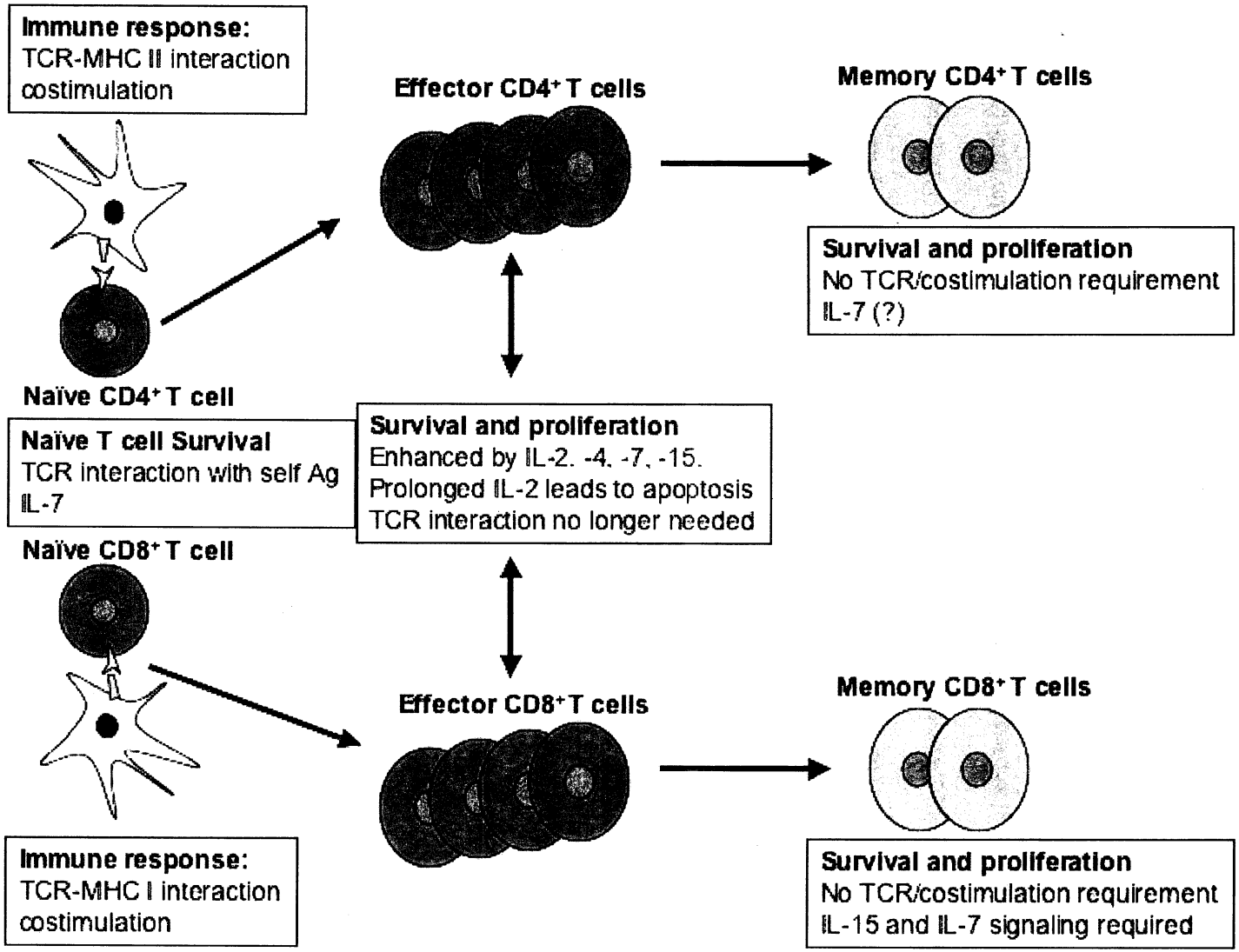
The periphery of $Jak3^{-/-}$ mice is characterized by the absence of Peyer's patches and nearly undetectable peripheral lymph nodes. Given the low numbers of thymocytes

generated in $Jak3^{-/-}$ mice, one might expect a reduction in the number of peripheral T cells. Yet, adult mice (>3 weeks of age) lacking Jak3 present normal numbers of peripheral αβ T cells [179-181]. Despite having apparently normal T cell numbers, closer examination reveals that T cell homeostasis is highly dysregulated. Most of the peripheral T cells are $CD4^+$ and appear to have an activated or memory-like phenotype, characterized by the expression of cell surface markers such as CD44. $CD8^+$ mature T cells are virtually absent from the periphery and also appear to have an activated or memory-like phenotype [179]. These observations, in conjunction with numerous other studies using mice deficient in single or double γ_c-dependent cytokines demonstrate, not only the importance of these cytokines for T cell homeostasis but also the difference in cytokine requirements for $CD4^+$ and $CD8^+$, as well as naïve and activated/memory T cell survival in the periphery [57, 73-75].

The survival of both naïve $CD4^+$ and $CD8^+$ T cells is dependent on the interaction of the TCR with self-MHC-self peptide complexes, as well as signals mediated through cytokine receptors, especially those that signal through the γ_c chain and Jak3 [57, 68, 70-73, 75]. Several studies have demonstrated that signals mediated by IL-7 but not others, are essential for the survival of naïve $CD4^+$ and $CD8^+$ T cells (Fig.5) [73, 75].

Fig.5 Requirements for mature T cell survival (adapted from [81])

Naïve $CD4^+$ and $CD8^+$ T cell survival is dependent on signals mediated by the IL-7R, as well as interactions between the TCR and self-MHC-self-peptide complexes presented by APCs. T cell activation is mediated by TCR recognition of antigenic peptides presented by self-MHC molecules on APCs, in conjunction with secondary signals initiated by specific interactions between costimulatory molecules expressed on mature APCs and T cells. T cell expansion following activation is enhanced by signals through cytokine receptors, such as IL-2R, IL-4R, IL-7R and IL-15R. Signals required for the production of memory $CD4^+$ and $CD8^+$ T cell populations remain unclear. The survival of $CD8^+$ memory T cells is dependent on signals regulated by IL-15 and IL-7. The mechanisms that mediate $CD4^+$ memory T cell survival are more controversial, however, recent reports suggest that memory $CD4^+$ T cell survival is dependent on IL-7-mediated signals.


Immune response:
TCR-MHC II interaction
costimulation
Effector CD4+ T cells
Memory CD4+ T cells
Survival and proliferation
No TCR/costimulation requirement
IL-7 (?)
Naïve CD4+ T cell
Naïve T cell Survival
TCR interaction with self Ag
IL-7
Survival and proliferation
Enhanced by IL-2, -4, -7, -15.
Prolonged IL-2 leads to apoptosis
TCR interaction no longer needed
Naïve CD8+ T cell
Effector CD8+ T cells
Memory CD8+ T cells
Immune response:
TCR-MHC I interaction
costimulation
Survival and proliferation
No TCR/costimulation requirement
IL-15 and IL-7 signaling required

In fact, T cells from IL-7R$^{-/-}$ mice have a shorter lifespan [56, 57]. However, given the importance of IL-7 signaling in thymocyte development it is possible that the mature T cells in these mice are intrinsically defective, leading to their inability to survive in the periphery. This is not a likely explanation since transferring naïve T cells into IL-7$^{-/-}$ hosts rapidly leads to their disappearance [73]. This observation is supported by results of experiments in which T cell survival was studied using thymectomized B6 mice that no longer possessed any means of T cell replenishment. The mice were injected with α-IL-7R mAb, blocking interactions between the receptor and IL-7 [75]. Under these conditions, naïve T cells were only able to survive for 2 or 3 weeks, as opposed to the long half life observed in T lymphocytes when the mice were not injected with the mAb [75]. The half life of 2 to 3 weeks was comparable to that observed for T cells in an MHC-deficient environment [195].

Furthermore, when naïve T cells are transferred into T cell-depleted mice injected with α-IL-7R mAb, homeostatic proliferation is blocked [75]. A decreased amount of Bcl-2 was observed in the T cell compartment of the α-IL-7R treated mice [195]. This was not the case when T cells were injected into IL-4$^{-/-}$ or IL-15$^{-/-}$ T cell depleted hosts [74]. Tan et al showed that naïve T cells cannot undergo homeostatic proliferation when transferred into T cell depleted IL-7$^{-/-}$ mice. However, addition of exogenous IL-7 restored their ability to proliferate [73]. One of the effects of IL-7 is the up-regulation of Bcl-2, although the restoration observed by Tan et al was not achieved when Bcl-2 transgenic donor T cells were transferred into IL-7$^{-/-}$ mice without the addition of exogenous IL-7. Therefore, while the upregulation of Bcl-2 may be one mechanism by

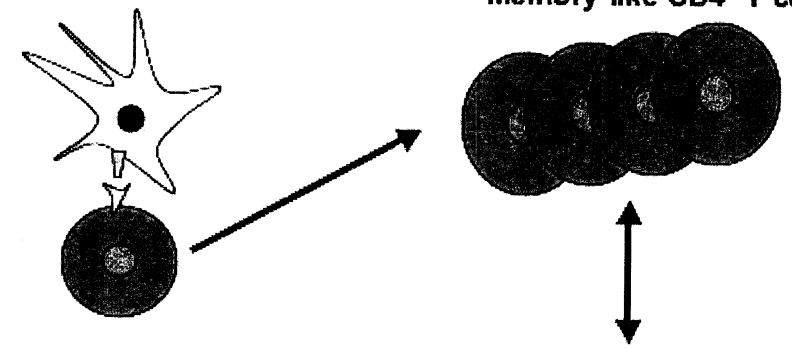
which IL-7 mediates the survival of naïve cells, other mechanisms are likely to play a role as well [73]. In contrast to these results, Seddon and Zamoyska showed that treatment with α-IL-7R mAb during the adoptive transfer of WT T cells into lymphopenic mice resulted in a considerable reduction of (especially in the $CD4^+$ T cell population), but did not completely block homeostatic proliferation of WT T cells [196]. They argued that both TCR and IL-7-mediated signals can independently induce expansion of T cells in a lymphopenic environment. Since total $CD4^+$ or $CD8^+$ T cells were used in these studies, it is possible that the proliferation observed in the absence of IL-7 could have been a result of memory $CD8^+$ or $CD4^+$ T cell homeostatic proliferation, which can take place independently of IL-7.

Finally, *in vitro* experiments using a system in which T-depleted whole lymphoid organs were cultured in media and injected with naïve T cells, also support an important role played by γ_c cytokines in T cell homeostasis. Briefly, CFSE-labelled naïve T cells were injected into the organs and T cell recovery, as well as homeostatic proliferation were measured after 7 days in culture. The results from these experiments showed that supplementing the media with IL-4, IL-7 and IL-15 increased the overall T cell recovery and enhanced homeostatic proliferation of the donor cells [73]. In contrast, supplementing the media with IL-2, IL-6 or IL-9 did not have an effect on homeostatic proliferation. When the cultures were supplemented with α-γ_c mAb, both the overall T cell recovery and homeostatic proliferation were significantly reduced. These data suggest that signals through Jak3/γ_c are essential for the survival of naive T cells, as well as homeostatic proliferation (Fig.6).

Fig.6 Requirements for mature T cell homeostatic proliferation (adapted from [81])

Mature T cells proliferate in a T cell depleted environment in order to reestablish normal T cell numbers. This phenomenon is known as homeostatic proliferation. Naïve $CD4^+$ and $CD8^+$ T cells require interactions between the TCR and self-MHC-self peptide complexes on APCs, as well as IL-7-mediated signals. Conversely, homeostatic proliferation of memory $CD4^+$ and $CD8^+$ T cells appears to be independent of MHC-TCR interactions in a lymphopenic environment. $CD8^+$ memory T cells require IL-15, and to a lesser extent, IL-7 signals to homeostatically proliferate. The requirements for $CD4^+$ memory T cells are unknown, but their ability to undergo homeostatic proliferation appears to be independent of γ_c cytokines.

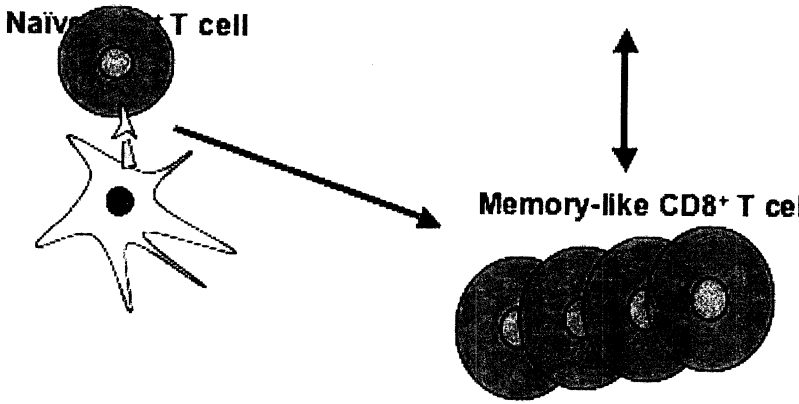
T CELL DEPLETED MOUSE



Naïve CD4+ T cell

Memory-like CD4+ T cells

Homeostatic Proliferation
TCR interaction (self antigen)
IL-7 (IL-15,-4,-12 enhance not required)
Space



Naïve CD8+ T cell

Memory-like CD8+ T cells

T CELL DEPLETED MOUSE

Memory CD4+ T cells

Homeostatic Proliferation
MHC independent
γc-cytokine independent
Space

Memory CD8+ T cells

Homeostatic Proliferation
MHC independent
IL-15 or IL-7
Space

In contrast to naïve T cells, the survival of memory T cells does not appear to be critically dependent on IL-7 but appears to be regulated by other γ_c cytokines. In particular, IL-15-mediated signals seem to be essential for the survival of $CD8^+$ memory T cells [74, 197]. Memory $CD8^+$ T cells express high levels of CD122 (IL-2Rβ), a component of the IL-15 and IL-2 receptors [198]. The presence of IL-15 leads to selective proliferation of $CD8^+$ memory T cells [198], while mice lacking either the IL-15 or IL-15R have reduced numbers of memory $CD8^+$ T cells [197, 199]. Additionally, experiments using IL-7R or IL-15R knock-out mice and IL-7R/IL-15R double knock-out mice suggest that IL-15 signals are necessary for the basal proliferation of memory $CD8^+$ T cells, while IL-7 signals are necessary for their survival [200]. $CD8^+$ memory T cell homeostatic proliferation appears to be dependent on similar factors. When $CD8^+$ memory T cells are transferred into T cell depleted IL-7 deficient mice, they proliferate normally. This proliferation is somewhat reduced when the cells are transferred into T cell depleted IL-15 deficient hosts. However, when $CD8^+$ memory T cells are transferred into IL-7/IL-15 deficient mice, homeostatic proliferation is virtually abolished [74, 200]. Therefore, it is not surprising that mice deficient in Jak3- or γ_c-mediated signals are also depleted of $CD8^+$ memory T cells. Contrary to these results, several studies using γ_c-deficient, as well as other single γ_c-dependent cytokine single knock-out mice had suggested that activated or memory $CD4^+$ T cells are independent of γ_c/Jak3 signals for survival [74, 75]. However, recent reports have shown that deficiencies in IL-7 lead to the disappearance of resting memory $CD4^+$ T cells and an inability to generate memory cells in lymphoid and non-lymphoid organs [201, 202]. Homeostatic proliferation of

memory $CD4^+$ T cells appears to take place normally in mice deficient in IL-7, IL-15 or both cytokines [74].

Collectively the data suggest that naïve $CD4^+$ T cells survive poorly in the absence of γ_c-dependent cytokine signals, whereas activated/memory $CD4^+$ T cell survival may be less dependent on these cytokines. Contrastingly, $CD8^+$ T cells, both naïve and memory, are highly dependent on γ_c-dependent cytokine signals and cannot survive in their absence.

Interestingly, when mice deficient in either Jak3 or γ_c chain are crossed to MHC Class I- or MHC Class II-restricted TCR transgenic mice, development appears to be similar between the two models and the thymi of $Jak3^{-/-}$ or $\gamma_c^{-/-}$ TCR transgenic mice are extremely reduced in cellularity [17, 203, 204]. However, unlike the straight knock-out mice, $Jak3^{-/-}$ or $\gamma_c^{-/-}$ TCR transgenic mice have very low numbers of mature T cells. This is true for both $CD8^+$ and $CD4^+$ TCR transgenic models. In addition, most of the T cells appear to have a naïve phenotype and those that present a memory-like phenotype do not express the transgenic TCR, but an endogenous α chain [203, 204]. Finally, our lab designed a mouse model in which Jak3 was expressed under the Lck promoter; T cells in these mice express Jak3 throughout development but lose its expression when they enter the periphery as mature cells [205]. Despite the fact that thymocyte development in these mice was normal, the peripheral phenotype was very similar to that of straight Jak3-deficient mice. As the mice aged and mature T cells lost Jak3 expression, the cells presented an activated or memory-like phenotype and the ratio of CD4 to CD8 was highly elevated.

Given the available data, it is not clear whether the dysregulation in T cell homeostasis observed in Jak3- or γ_c-deficient mice is a consequence of developmental defects or a consequence of peripheral activation and proliferation. Also, we do not know if the phenotype observed in the periphery of these mice may be rescued by introducing a survival factor to be expressed by developing thymocytes and mature T cells.

C.3 Jak3/γ_c signals in T cell activation

In addition to the importance of γ_c/Jak3-mediated signals in the maintenance of T cell homeostasis, these signals also play an important role in T cell activation, proliferation and differentiation. Although $CD4^+$ $CD44^{hi}$ T cells from mice deficient in γ_c/Jak3 appear to have an activated or memory phenotype, they respond extremely poorly to stimulation *in vitro*. When splenocytes from Jak3-deficient mice were cultured in the presence of concavalin A, anti-CD3, anti-CD3 plus anti-CD28, or PMA plus ionomycin, their proliferation was minimal [179]. On average, the responses from both thymocytes and splenocytes from $Jak3^{-/-}$ mice were less than 10% the response of wild type control cells. Additionally, stimulated splenocytes from Jak3-deficient mice secreted less IL-2 than wild type cells [179]. All of these experiments were conducted using unfractionated $CD4^+$ T cells from the spleens of adult mice. The total numbers of $CD44^{low}$ $CD4^+$ T cells in the periphery of these mice are so minimal that it has previously been impossible to conduct experiments using naïve $CD4^+$ T cells from straight Jak3 or γ_c chain knock-out mice. Similarly, the total numbers of naïve as well as memory-like $CD8^+$ T cells in the

periphery of Jak3/γ_c-deficient mice are extremely reduced, making it impossible to study their ability to respond to an antigenic challenge *in vivo* or *in vitro*. Thus, the requirements for these cytokines to initiate, sustain, and contract immune responses are still unclear.

C.3.a $CD4^+$ T cell activation in the absence of γ_c chain cytokine signaling

In trying to elucidate the role played by γ_c cytokines in the activation of T cells, Di Santo's group designed experiments in which they compensated for the lack of naïve $CD4^+$ T cells in the periphery of γ_c-deficient mice by crossing these mice to TCR transgenic mice [206]. The transgenic model used by this group was a $CD4^+$ TCR specific for the H-Y male antigen. As mentioned before, when Jak3- or γ_c-deficient mice are crossed to MHC Class I- or Class II-restricted TCR transgenic mice, their periphery is characterized by very low numbers of naïve T cells, once more emphasizing the essential role of Jak3/γ_c-mediated signals for the survival of these cells, and the absence of memory-like T cells that populate the periphery of the straight knock-out mice. However, they were able to pull enough mature thymocytes from γ_c^- TCR transgenic mice, as well as γ_c^+ control thymocytes and adoptively transfer equal numbers of each phenotypic model into wild type lymphopenic mice. They observed that the γ_c^+ TCR transgenic (TCR^+) cells survived for extended periods of time in the periphery of alymphoid female mice, while the γ_c^- TCR^+ cells were lost five days after transfer. When γ_c^- TCR^+ mice were injected with male CD3ε-deficient splenocytes that carry the specific epitope recognized by the transgenic TCR, the $CD4^+$ T cells clonally expanded to levels

equivalent to that of the γ_c^+ control T cells, with similar kinetics of activation. Additionally, when a mixture of 80% γ_c^+ and 20% γ_c^- naïve TCR^+ T cells was injected into the periphery of male alymphoid mice, the extent of proliferation and activation was similar between the two groups of cells. Finally, in order to investigate the ability of γ_c^- T cells to become memory cells, they transferred previously primed γ_c^+ TCR^+ and γ_c^- TCR^+ cells into alymphoid female hosts. Both γ_c^+ and γ_c^- T cells persisted for at least 5 weeks, and upon antigenic re-challenge, they both proliferated extensively. Therefore, their data suggested that signals through the γ_c chain were necessary for naïve T cell survival but not for antigen proliferation, or for the maintenance and restimulation of memory $CD4^+$ T cells [206].

C.3.b $CD8^+$ T cell activation in the absence of IL-2-mediated signals

Additional experiments to better understand the role of γ_c cytokines during an immune response have been conducted using mice that are deficient in individual cytokine or cytokine receptors of the γ_c family. The results from these investigations are somewhat controversial. Some of the studies have concurred with the observation that these deficiencies have relatively minor effects on T cell activation and function. Several *in vitro* studies indicate that IL-2 is a major T cell growth factor, and that it plays a critical role in the differentiation of naive T cells into effector cells. Yet, when IL-2-deficient mice were infected with vaccinia virus, they were able to generate protective immunity to the virus, producing both CTL and anti-viral antibody responses [207]. Infection of IL-2-deficient mice with lymphocytic choriomeningitis virus (LCMV)

resulted in a three-fold reduction of the $CD8^+$ T cell response, but the mice were able to clear the virus by day 10. This particular study also demonstrated that, unlike the results obtained *in vivo*, when $CD8^+$ T cells were stimulated *in vitro* they were not able to differentiate into CTLs in the absence of IL-2. IL-4 could somewhat compensate for the lack of IL-2 *in vivo* but not *in vitro*. The results suggested that a network of cytokines, other than those tested *in vitro*, which included IL-1, IL-4 and IL-6, might play a compensating role in IL-2-deficient mice. Concurring with these results, when studies were performed using an IL-2R$\beta^{-/-}$ mouse model, in which both the IL-2 and IL-15 high affinity-signals are blocked, Yu et al observed that $CD8^+$ T cells were able to induce an efficient response *in vivo*, although the magnitude and effector responses were somewhat impaired [208]. They observed that the amount of IFNγ secreted by cells from these mice in response to a vaccinia infection was markedly reduced, in addition to their inability to elicit a CTL response *in vitro*, due to reduced induction of perforin and granzyme B. [208]. Other studies in which IL-2-deficient mice were crossed to an influenza-specific TCR transgenic background suggested that, in the absence of the cytokine, $CD8^+$ T cells were able to proliferate and develop a blastogenic response, but Ag-specific cytotoxicity was only detected in animals that were able to produce IL-2 [209].

Somewhat contrasting with these conclusions, additional studies in which IL-2-deficient mice were also infected with LCMV suggested that, in the absence of the cytokine, the expansion of $CD8^+$ T cells following viral infection was dramatically inhibited [210]. The percentages of $CD8^+$ T cells did not increase unless IL-2 was added

during the infection. Further, the production of IFN-γ was significantly reduced at the peak of the immune response in IL-$2^{-/-}$ mice, but the exogenous addition of IL-2 was enough to restore the ability of these mice to produce normal levels of IFN-γ in response to LCMV [210]. Recent studies, using the OT-1 TCR transgenic model, have suggested that in the absence of IL-2, $CD8^+$ T cells can initiate a specific immune response but they cannot undergo the subsequent proliferative expansion [211]. Briefly, OT-1^+ $CD8^+$ T cells from WT, IL-$2^{-/-}$, or IL-2Rα$^{-/-}$ mice were labeled with CFSE and adoptively transferred into normal mice. The mice were then injected with a VSV-OVA construct and the immune response was followed during a time course. Early in the infection the magnitude of proliferation between the WT and IL-$2R^{-/-}$ cells was comparable. However, continued expansion of the IL-$2R^{-/-}$ cells was impaired after the first 3 days of infection [211]. Addition of exogenous IL-2 was enough to sustain the proliferative phase inhibited in IL-2-deficient $CD8^+$ T cells. Therefore, it was concluded that the initial $CD8^+$ T cell division following an immune challenge is IL-2 independent, while the proliferation subsequent to activation was IL-2 dependent. Similarly, studies in which IL-15 knock-out mice were infected with VSV resulted in a reduction of tetramer positive $CD8^+$ T cells compared to the wild type controls. The reduction was only obvious after day 6 of infection, demonstrating that IL-15 was important in determining the amplitude of the VSV-specific primary response [212]. These mice were also deficient in generating a substantial portion of antiviral $CD8^+$ memory T cells [212]. The percentage of Ag-specific IL-$15^{-/-}$ $CD8^+$ T cells 15 days after VSV infection was only ~40% of the WT control levels, and 77 days after infection it was only ~23%. However, a subset of

long-lived $CD8^+$ memory T cells survived in the absence of IL-15. One possibility is that the IL-15-independent memory T cell population is maintained by IL-7-mediated signals.

C.3.d Activation induced cell death in the absence of γ_c cytokine signaling

An important step following T cell activation is the elimination of activated T cells, once the infection has been cleared. Due to the high percentage of activated $CD4^+$ T cells present in the periphery of γ_c/Jak3 deficient mice, Leonard's group went on to investigate the ability of these mice to undergo T cell AICD following an immune response [203]. They injected $\gamma_c^{-/-}$ mice with a super antigen, SEB, and compared the $CD4^+$ T cell response to that of wild type control mice. Even though $\gamma_c^{-/-}$ $V_\beta 8$ $CD4^+$ T cells expanded less that those in wild type mice in response to SEB, some expansion was observed. This suggested that γ_c cytokines are required for a full response but also that γ_c-independent signals can mediate a certain level of expansion. The SEB-induced expansion was followed in wild type mice by a deletion of $V_\beta 8$ $CD4^+$ T cells, but not in $\gamma_c^{-/-}$ mice. These results suggested that clonal deletion is impaired in γ_c/Jak3 deficient mice. Additionally, they observed that expression of FasL was significantly decreased in activated $\gamma_c^{-/-}$ $CD4^+$ T cells. However, when activated $CD4^+$ T cells from both $\gamma^{-/-}$ and wild type mice were incubated in the presence of α-Fas Ab, γ_c^- $CD4^+$ T cells were much more susceptible to Fas mediated death. Taken together with the accumulation of activated T cells, the results suggest that γ_c-deficient activated T cells are not deleted by the Fas-FasL pathway, due to an impaired expression of FasL [203].

Although some studies have been conducted using γ_c-deficient $CD4^+$ T cells, very little is known about the effect that complete lack of signals mediated by all the γ_c cytokines would have in the ability of $CD8^+$ T cells to mount a proper complete immune response. Due to the lack of viable $CD8^+$ T cells in the absence of Jak3/γ_c signaling, it has not been possible to conduct these studies. The first step should be to infect Jak3 deficient mice with a well characterized virus, and compare their response to that of wild type control mice. However, as mentioned before the numbers of $CD8^+$ T cells present in the periphery of $Jak3^{-/-}$ mice are highly reduced, leading to the likelihood that these mice do not have enough cells to mount a response, regardless of whether the $CD8^+$ T cells themselves are capable of responding. Ideally, the same number of wild-type or Jak3 deficient $CD8^+$ T cells should be stimulated, using a specific antigen, following a direct comparison of both responses over a time course.

D. WORK PRESENTED IN THIS THESIS

The overall goal of my thesis was to obtain further knowledge on the role that cytokines play in the maintenance and function of the immune system. More specifically I investigated the role played by cytokines of the γ_c family in thymocyte development, T cell homeostasis and $CD8^+$ T cell function. Using the Jak3-deficient mice as a model, we investigated the following aims:

1. Chapter three: "Two distinct mechanisms lead to impaired T cell homeostasis in Janus Kinase 3- and CTLA-4-deficient mice". Given the presence of activated $CD4^+$ T cells in the periphery of Jak3-deficient mice, we investigated whether this activation was initiated

by an Ag-mediated mechanism, or was a result of an intrinsic T cell defect, independent of Ag activation. We compared the Jak3-deficient mouse model to the CTLA-4-deficient mouse model that presents a similar dysregulation in T cell homeostasis. Using spectratype analysis we concluded that the TCR repertoire of $Jak3^{-/-}$ mice appeared to be skewed, suggesting that the activation of CD4 T cells observed in the periphery of these mice was Ag-mediated. Contrary to these results, the TCR repertoire of $CTLA\text{-}4^{-/-}$ mice appeared to be highly diverse, suggesting that the activation and proliferation of $CD4^+$ T cells observed in the periphery of these mice was Ag-independent and may be a result of a T cell intrinsic defect. We conclude that two very similar phenotypes of peripheral T cell homeostasis dysregulation are clearly derived by distinct mechanisms.

2. Chapter four: "T cell defects in Jak3-deficient mice are not rescued by the enforced expression of Bcl-2". In this chapter we investigate the ability of Bcl-2, an antiapoptotic molecule, to rescue thymocyte development and T cell homeostasis in Jak3-deficient mice. Previous studies had investigated the role played by Bcl-2 in IL-7-deficient and γ_c-deficient mice, but the results remained highly controversial. In our hands, enforced expression of Bcl-2 did not rescue the block observed in the development of thymocytes that lack Jak3/γ_c-mediated signals. Furthermore, the periphery of these mice was populated by mostly activated T cells, with a significantly elevated CD4 to CD8 ratio. Enforced expression of Bcl-2 did rescue a significant population of mature naïve CD8 T cells when the mice are crossed to OT-1 TCR transgenic mice. Therefore we conclude that Bcl-2 expression does not rescue T cell development or T cell homeostasis in Jak3-

rescue T cell development or T cell homeostasis in Jak3-deficient mice, but can mediate the survival of a mature T cell population when the mice are in a TCR transgenic background.

3. Chapter five: "Antiviral immune responses are initiated but not sustained by T cells lacking Jak3-mediated cytokine signals". In this chapter we investigate the ability of CD8 T cells to mount an immune response against two different viruses, in the absence of Jak3-mediated signals. $Jak3^{-/-}$ and $Jak3^{+/-}$ control mice were infected with LCMV and their responses were compared over a time course. Additionally, OT-1^{+}bcl-2^{+} $CD8^{+}$ T cells with or without Jak3 were specifically activated *in vitro*, and *in vivo* after being adoptively transferred into WT congenic mice. The results from both systems are somewhat consistent and they suggest that $CD8^{+}$ T cells can initiate a limited immune response against a viral challenge, but are incapable of sustaining the response or clearing the infection.

CHAPTER II

MATERIALS AND METHODS

Mice

In chapter III, $Jak3^{-/-}$ and $Jak3^{+/-}$ mice [179, 181], $CTLA4^{-/-}$ and $CTLA4^{+/-}$ mice [179], which have been described previously, were used for analysis of TCR repertoire. Mice were backcrossed to C57BL/10 or C57BL/6, respectively, a minimum of four generations. $Jak3^{-/-}$ mice and littermate controls were 8-9 weeks of age. $CTLA\text{-}4^{-/-}$ and littermate controls were 2 weeks of age. All experiments using $Jak3^{-/-}$ and $CTLA\text{-}4^{-/-}$ mice were conducted using their respectively littermates on the same day. Male C57BL/6 mice (Jackson laboratories, Bar Harbor, ME) were used for the LCMV infections. In chapter IV, $Jak3^{-/-}$ mice were crossed to Eμ-Bcl-2-36 transgenic mice [C57BL/6-Tg (Bcl-2) 36Wehi] [213] (Jackson laboratories, Bar Harbor, ME). $Jak3^{-/-}bcl\text{-}2^{+}$ and $Jak3^{+/-}bcl\text{-}2^{+}$ controls, as well as straight $Jak3^{-/-}$ and $Jak3^{+/-}$ control mice were all littermates 6-10 weeks of age. $Jak3^{-/-}$ or $Jak3^{+/-}bcl\text{-}2^{+}$ mice were crossed to OT-1 TCR transgenic mice [33], kindly donated by Dr. Kenneth Rock's laboratory. In chapter V, $Jak3^{-/-}$ or $Jak3^{+/-}bcl\text{-}2^{+}$ $OT\text{-}1^{+}$ mice were used for the adoptive transfer experiments and infections with the vaccinia-ova construct. All mice and their controls were littermates 8-10 weeks of age. Congenic Ly5.2 C57BL/6 (NCI-Frederick, Fort Detrick, MD) 8-10 weeks old mice were used as hosts for the adoptive transfer experiments. These mice are genetically identical to C57BL/6 mice except for the Ly5.1 allele. $Jak3^{-/-}$ mice backcrossed to C57BL/6 (Jackson laboratories, Bar Harbor, ME) and $Jak3^{+/-}$ littermate control mice or C57BL/6 control mice were used for the LCMV infections. All mice used for the experiments were maintained in a specific-pathogen-free facility.

Antibodies and Flow Cytometry

For antibody staining experiments, splenocytes were isolated and depleted of red blood cells. Cells were then stained with the indicated antibodies in HBSS supplemented with 3% FCS for 20 minutes on ice. Samples were analyzed on a Becton Dickinson FACSCalibur (Becton Dickinson, San Diego, CA) using the CellQuest software (Becton Dickinson). The antibodies used were the following: in chapter III, α-CD44-FITC (clone IM7), α-CD69-FITC (clone H1.2F3), α-CD4-PE (clone H129.19), α-CD8-PerCP (clone 53-6.7), α-CD8-FITC (clone 53-6.7) (all obtained from Pharmingen, San Diego, CA), and α-CD62L (clone MEL 14), (obtained from e Bioscience, San Diego, CA); in chapter IV, biotinylated Abs. (α-CD3, α-CD4, α-CD8, α-B220, α-Gr-1, α-IgM, α-Ter119, α-Mac1, and α-DX5), streptavidin-cy, α-CD25 (IL-2Rα, p55)-PE, αCD44 (Ly-24)-FITC, α-CD8 (Ly-2)-APC, α-CD4-PE, α-CD62-L (MEL-14)-cy, α-Bcl-2-bio, α-Vα2 (B20.1)-PE, α-Vβ8 (CMR5-2)-FITC (obtained from Pharmingen, San Diego, CA); in chapter V, α-CD16/CD32 (Fcg III/II receptor, clone 2.4G2), α-CD8-cy, α-CD4-FITC, α-IFN-γ-APC, α-IFN-γ-PE, α-IL-2-PE, α-TNF-α-APC, α-IgG_1-APC, α-IgG_{2b}-PE, α-CD45.2-bio (obtained from Pharmingen, San Diego, CA) and α-human granzyme B-PE (obtained from Caltech Laboratories, Burlingame, CA). In chapter III $CD4^+$ thymocytes were sorted on a Becton Dickinson FACSTAR® to 94% purity.

Virus Stocks and Immunization

In chapters III and V, mice were infected with LCMV. LCMV, strain Armstrong, an RNA virus in the Old World arenavirus family, was propagated in BHK21 baby

hamster kidney cells [214]. 4 X 10^4 plaque forming units of the virus were used to infect mice intraperitoneally. Splenocytes were isolated at different time points after infection.

RNA Extraction and cDNA synthesis

For experiments described in chapter III, total RNA was isolated from whole spleens and thymi using TRIZOL as described by the manufacturer (Life Technologies Inc., Grand Island, NY). Briefly, 2 ml of TRIZOL was used for the thymi of $Jak3^{+/-}$ mice, spleens of $Jak3^{+/-}$ and $Jak3^{-/-}$ mice, and spleens of $CTLA4^{+/-}$ and $CTLA4^{-/-}$ mice, while 1 ml of TRIZOL was used for the thymi of $Jak3^{-/-}$ mice, and for 1-10 x 10^6 sorted thymocytes. RNA was resuspended in 10-15 µl of DEPC water. Total cDNA was synthesized from an average of 4 µl of RNA using the Pharmacia Kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ).

CDR3-Length Spectratyping

A detailed protocol has previously been described [29, 215]. Briefly, 3µl of cDNA was subjected to PCR amplification using a C_β primer and one of the V_β primers. Eight different V_β primers; V_β16 (C ACT CTG AAA ATC CAA CCC AC), V_β14 (AC GAC CAA TTC ATC CTA AGC AC), V_β11 (G CAC TCA ACT CTG AAG ATC CAG AGC), V_β10 (ATC AAG TCT GTA GAG CCG GAG GA), V_β8 (CAT TAC TCA TAT GTC GCT GAC), V_β6 (CT CTC ACT GTG ACA TCT GCC C), V_β5.2 (AAG GTG GAG AGA GAC AAA GGA TTC), and V_β5.1 (CAT TAT GAT AAA ATG GAG AGA GAT) [215] were used to analyze splenocyte samples from 5 $Jak3^{-/-}$ mice and 3 littermate

Jak3$^{+/-}$ controls. Splenocyte samples from an additional 2 Jak3$^{+/-}$ and 2 Jak3$^{-/-}$ mice, plus 4 CTLA4$^{-/-}$ and 4 littermate CTLA4$^{-/-}$ mice, were analyzed with four V_β primers (V_β11, V_β10, V_β8, V_β5.2). The PCR products were subjected to run-off reactions using 6 fluorophore-labeled J_β primers; J_β1.1 (XAC TGT GAG TCT GGT TCC TTT ACC), J_β1.2 (XA AAG CCT GGT CCC TGA GCC GAA G), J_β2.1 (XGT GAG TCG TGT TCC TGG TCC GAA G), J_β1.3 (XCT TCC TTC TCC AAA ATA GAG C) J_β1.4 (XGA CAG CTT GGT TCC ATG ACC G), and J_β1.5 (XG AGT CCC CTC TCC AAA AAG CG) [215], synthesized by Applied Biosystems Inc., Foster City, CA. The products were loaded onto a 4.57% acrylamide sequencing gel and the results were analyzed on an automated DNA sequencer using GeneScan software (Perkin-Elmer Applied Biosystems, Emeryville, CA).

Generation of dendritic cells for *in vitro* stimulations

For chapter V bone marrow was isolated from femurs of C57BL/6 mice and subjected to red blood cell lysis. The cells were resuspended at 7x10^6 cells per plate in 15 mL of media (10% FCS, 1X PSQ, 1X βme, 2.5mM HEPES) containing 150μg GM-CSF and 75μg IL-4. The cells were incubated ay 37° for 8 days. On day 8, dendritic cells were collected from plates and resuspended at 1.0X10^6 cells in 300uL of 10% FCS media containing different concentrations of SIINFEKL peptide. The cells were incubated for 45 min. at 37°. Cells were washed with 2% FCS HBSS (without Ca^{2+}/Mg^{2+}) and then incubated with mytomicin c (50μg/mL) for 45 min. at 37°. Cells

were washed with 10% FCS media and resuspended at $5.0x10^5$ cells/mL. 100μL ($5.0x10^4$ cells) were used per well in 96 well plate.

Purification of $CD8^+$ T cells

For chapter V, single cell suspensions from spleens of $Jak3^{+/-}$ or $Jak3^{-/-}$bcl-2^+OT-1^+ littermate mice were prepared. After red blood cell lysis, $CD8^+$ cells were positively selected by incubation with α-CD8 (Ly2) coated microbeads (Miltenyi Biotec) followed by passage through a LS^+ columns according to manufacturer's protocol (Miltenyi Biotec, Auburn, CA. Purified cells were used for *in vivo* and *in vitro* experiments. For *in vivo* experiments, purified $CD8^+$ T cells were pooled and labeled with CFSE. A sample of the positive fractions was stained with α-Vα2 and α-CD8 to test for purity. $CD8^+$ T cells were resuspended in sterile 1X PBS at $1.2X10^7$ cells/mL. For *in vitro* experiments, purified $CD8^+$ T cells were pooled and resuspended in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 2mM L-glutamine, 100U penicillin, 100μg/mL streptomycin, 10mM HEPES, and 50 μM β-ME. A sample of each positive fraction was stained with α-CD8 and α-Vα2 Abs. and checked for purity. $CD8^+$ T cells were labeled with CFSE. The cells were resuspended at $5.0x10^5$ $CD8^+$ $V\alpha2^+$ cells/mL in 10% FCS media.

CFSE labeling

Purified $CD8^+$ $V\alpha2^+$ T cells were labeled with CFSE using a protocol previously described [216]. Briefly, the purified cells were suspended in HBSS at 2.5 x 107 cells/ml

and incubated in 2 μM CFSE (Molecular Probes, Eugene, OR) solution for 15 min at 37°C. After incubation, donor cells were washed twice with HBSS and resuspended in either 1X PBS (adoptive transfers) or 10% RPMI media (*in vitro* stimulations).

***In vitro* Proliferation Assays**

For stimulations, 5.0 X 10^4 purified $CD8^+$ $V\alpha2^+$ cells were incubated with SIINFEKL peptide plus 5.0 X 10^4 mitomycin-treated dendritic cells in a volume of 200μL for 24 or 48 hours. For a control, cells were also stimulated with PMA (Sigma; 2.5ng/mL) and Ionomycin (Calbiochem; 375ng/mL). ^{3}[H] thymidine (NEN, Boston, MA) was added at 1μCi/well and incubated for an additional 20 hours at 37°. The plates were harvested on a Tomtec harvester 96 and ^{3}[H] thymidine incorporation was quantified on a Perkin Elmer trilux microbeta counter.

Adoptive transfers

Purified $CD8^+$ $V\alpha2^+$ T cells from $Jak3^{+/-}$ or $Jak3^{-/-}$bcl-2^+OT-1^+ mice were labeled with CFSE as described above. 2.5x10^6 $CD8^+$ $V\alpha2^+$ cells were injected intravenously into congenic Ly5.2 C57BL/6 mice.

vaccinia-ova injections

24 hours after the injection of $CD8^+$ $V\alpha2^+$ T cells, host congenic mice were injected with 1.0x10^7 PFUs of a vaccinia-ova construct intravenously. The vaccinia-ova

construct, kindly donated by Dr. Kenneth Rock's laboratory, has previously been described [217]

Intracellular cytokine staining

In chapter IV, single cell suspensions from total thymocytes and splenocytes from $Jak3^{+/-}$, $Jak3^{-/-}$, $Jak3^{+/-}bcl\text{-}2^{+}$ and $Jak3^{-/-}bcl\text{-}2^{+}$ mice were prepared. After lysis of red blood cells, all cells were stained for 20 minutes on ice, fixed for 20 minutes, permeabilized and stained intracellularly with α-Bcl-2-bio Ab according to the Cytofix/Cytoperm™ kit protocol (Pharmingen). Cells were immediately analyzed by flow cytometry on a BD FACSCalibur. In chapter V, single cell suspensions of the spleens from congenic mice injected with the $Jak3^{+/-}$ or $Jak3^{-/-}bcl\text{-}2^{+}OT\text{-}1^{+}$ cells and with or without the Vac-OVA construct were generated. After RBC lysis $2.0x10^{6}$ splenocytes were incubated with or without PMA (10ng/mL) and Ionomycin (400ng/mL) in the presence of Golgi plug for 5 hours at 37°. After incubation the cells were washed and stained for 20 minutes, fixed, then permeabilized and stained with α-IFN-γ-APC, and α-IL-2-PE, or α-IgG-APC, or α-IgG-PE according to the Cytofix/cytoperm™ kit protocol (Pharmingen). Cells were immediately analyzed by flow cytometry. For the LCMV infection, D0, D3, D5, D8, D14 or one month after LCMV infection of $Jak3^{+/-}$ or Jak3-/- mice, splenocytes were isolated and single cell suspensions were generated. Total splenocytes were incubated in 96 well plates at 2.0×10^{6} cells/well in a 100μL volume. The cells were incubated with 5μM synthetic peptide, and 0.2μL Golgi Plug™ (Pharmingen) for 5 hours at 37°. All cells were stained for 20 minutes, fixed for 20

minutes, and permeabilized and stained intracellularly with α-IFN-γ-APC, α-IL-2-PE, α-TNF-α-APC or α-IFN-γ-PE. Cells were also stained with isotype control Abs. according to the Cytofix/cytoperm™ kit protocol (Pharmingen). Peptides used here have been previously described [218, 219]. They include NP396–404 (FQPQNGQFI), gp33–41 (KAVYNFATC), NP205–212 (YTVKYPNL), GP61-80 (GLNGPDIYKGVYQFKSVEFD). They were purchased from American Peptide (Sunnyvale, CA) at 90% HPLC purity.

Tetramer Staining

Peptide-loaded H-2K^b and H-2D^b tetramers were prepared as previously described. For staining, cells were first blocked against non-specific binding with α-FcR Ab. Samples were washed and then co-stained with the indicated peptide-loaded tetramer and anti-CD8 for 1 hr at 4°C. Samples were then washed twice and fixed with Cytofix™ solution (BD PharMingen). Cells were immediately analyzed by flow cytometry.

Virus Titrations

Infectious LCMV was quantified by plaque assay, as previously described [220]. Briefly, spleens were ground in RPMI and centrifuged for 20 minutes to pellet off cell debris. The samples were frozen at –20°. One day before the assay 1.0 X 10^5 Vero cells were plated in 35-mm wells in 6-well dishes (Costar, Cambridge, MA). The plates were incubated at 37°C and used the following day for the assay when the cell monolayers were confluent. The medium was removed and titrations of infected spleen samples

previously frozen down were added to the cells (0.2 ml vol.). After adsorption for 90 min at 37°C, the cells were overlaid with 4 ml of a 1:1 mixture of 0.8% Seakem agarose (FMC Corporation, Rockland, ME) in EMEM (Gibco Laboratories, Grand Island, NY) supplemented with 5% heat-inactivated fetal calf serum, antibiotics, and L-glutamine. The plates were incubated for 5 d at 37°C and then overlaid with 2.0 ml of a 1:1 mix of 0.8% agarose and EMEM complete, containing 15 μL of 1% neutral red (Gibco Laboratories) per mL of mix. Plaques were counted the following day.

CHAPTER III

TWO DISTINCT MECHANISMS LEAD TO IMPAIRED T CELL HOMEOSTASIS IN JANUS KINASE 3- AND CTLA-4-DEFICIENT MICE

A. INTRODUCTION

One of the most fascinating aspects of the immune system is the maintenance of T cell homeostasis. T cell development in the thymus continues throughout life, generating new mature naïve T cells, with a highly diverse TCR repertoire. Despite this continual export of new lymphocytes from the thymus, in the absence of infectious agents, peripheral T cell numbers are maintained at a constant, tightly regulated level. When $CD4^+$ and $CD8^+$ T cells encounter their specific antigen in the periphery, they become activated and proliferate as they differentiate into effector cells [52, 97, 102]. This dramatic expansion in the number of antigen-specific T cells is essential for a successful immune response. However, to maintain constant T cell numbers this expansion must be immediately followed by the specific contraction of activated T cells, accompanied by the emergence of a small memory T cell population. Therefore, under normal circumstances, the peripheral T cell compartment of healthy mice is comprised of at least three different subsets of T cells; recent thymic emigrants, resident naïve T cells, which are maintained in constant numbers, with a constant $CD4^+$ to $CD8^+$ T cell ratio, and a population of antigen experienced memory T cells.

The maintenance of these T cell populations is crucial for the adequate function of the immune system, and dysregulations in lymphoid homeostasis may lead to the inability of the system to mount functional immune responses and could, under certain circumstances, lead to death. Mice deficient in Jak3 or CTLA-4 are among the most dramatic examples of T cell homeostatic dysregulation. These two models will be discussed is more detail in this chapter.

Given the importance of lymphoid homeostasis, many studies have attempted to define the factors that contribute to homeostatic regulation. It has been established that each of the different peripheral lymphocyte populations are independently regulated and are dependent on different factors to maintain their homeostatic state. It has also been shown that in the absence of normal peripheral T cell numbers, mature naïve $CD4^+$ and $CD8^+$ T cells will proliferate until normal T levels are restored. Following this process, known as homeostatic proliferation, T cells acquire an activated/memory-like phenotype, characterized by the upregulation of CD44. Although it is unclear exactly what triggers this proliferation, it is known that different mechanisms support the proliferation of the different populations (naïve $CD4^+$ and $CD8^+$, and memory $CD4^+$ and $CD8^+$)

Among the factors that contribute to the maintenance of T cell homeostasis are cytokines, and of special importance are the cytokines that signal through the γ_c chain. Several studies have demonstrated that signals mediated by IL-7 but not others, are essential for the survival of naïve $CD4^+$ and $CD8^+$ T cells. In fact T cells from IL-$7R^{-/-}$ mice have been found to have a shortened lifespan, and when normal naïve T cells are transferred into IL-$7^{-/-}$ hosts they also disappear rapidly [56, 57, 73]. In addition, naïve T cells transferred into T cell depleted mice in the presence of α-IL-7RmAb, or transferred into T cell depleted IL-$7^{-/-}$ mice, cannot undergo homeostatic proliferation, [75], but addition of exogenous IL-7 restores their ability to proliferate [73]. The role of cytokines in maintaining homeostasis of naïve T cells is also supported by *in vitro* data. The survival of naïve T cells, and their ability to undergo homeostatic proliferation was increased by supplementing culture medium with IL-4, IL-7 and IL-15, while culturing

naïve T cells in the presence of mAbs specific for the γ_c chain, decreased their survival rate dramatically [73]. The dependence of naïve T cells on IL-7 for survival may be mediated by Bcl-2 induction, as has been suggested by both *in vitro* and *in vivo* studies [221-223].

The survival of memory T cells appears to be regulated by different cytokines. Signals mediated through IL-15 (also a γ_c cytokine) and to a lesser extent IL-7, appear to be essential for the survival of $CD8^+$ memory T cells [74, 197, 200]. The factors that mediate the survival of CD4 memory T cells, including cytokines, are not well defined. Several reports had previously suggested that their survival was independent of γ_c/Jak3 signals [74, 75], but more recent studies suggest that they may actually require IL-7-mediated signals to survive [201, 202].

A.1 $Jak3^{-/-}$ and CTLA-$4^{-/-}$ mouse models of dysregulated T cell homeostasis

In recent years, the generation of mouse lines with specific genetic deficiencies have provided some insights into the regulation of T cell homeostasis. Two such models of dysregulated T cell homeostasis are mice deficient in Jak3, and mice lacking the T cell costimulatory molecule, CTLA-4. Mice deficient for either Jak3 or CTLA-4 present a similar phenotype characterized by an apparently polyclonal expansion of the peripheral T cells. However, it is not known whether similar mechanism(s) are responsible for this expansion. Understanding the basis of the phenotype observed in these animals would provide important insights into the roles of Jak3 and CTLA-4 in T cell homeostasis.

Jak3 is preferentially expressed in hematopoietic cells, where it is associated with the cytokine receptor common γ-chain (γ_c), a component of the receptors for IL-2, -4, -7, -9, and –15 [224]. Deficiencies in Jak3 lead to severe combined immunodeficiency (SCID) condition in humans and mice. Specifically, $Jak3^{-/-}$ mice are characterized by a block in B, NK, and γδ T cell development [179, 180, 189, 191, 193]. Although there is a reduction in the cellularity of the thymus, αβ thymocyte development appears to progress normally. Despite this, $Jak3^{-/-}$ mice have plentiful numbers of peripheral T cells, but they are predominantly $CD4^+$, with a virtual absence of mature $CD8^+$ T cells. Further, these cells resemble activated and/or memory cells in that they are large and express surface markers ($CD44^{hi}$, CD25, CD69) characteristic of prior activation [179-181, 204]. These T cells expand in the periphery, leading to an increase in overall T cell numbers. Interestingly, when TCR transgenic mice are crossed with $Jak3^{-/-}$ mice, the peripheral T cell pool is dramatically reduced in numbers and the cells remain phenotypically naïve ($CD44^{lo}$, $CD25^{lo}$, $CD69^{lo}$) [204, 225].

CTLA-4 is a CD28 homologue that acts as a negative regulator of T cell activation. Interaction of CTLA-4 with its ligands, B7-1 and B7-2 (CD80 and CD86), inhibits T cell proliferation and reduces TCR and CD28 signaling during T cell activation [101]. Unlike the $Jak3^{-/-}$ mice, CTLA-$4^{-/-}$ mice have normal thymocyte numbers and development [226], yet the peripheral T cell phenotype of the two mouse strains is remarkably similar. Peripheral CTLA-$4^{-/-}$ T cells are predominantly $CD4^+$, and virtually all of them express activation markers ($CD44^{hi}$, CD25, CD69). Furthermore, the mice die at about three weeks of age due to a lymphoproliferative disorder [179, 227]. In contrast

to the findings with Jak3$^{-/-}$ TCR transgenic mice, this disease process can be delayed, but not prevented, by introducing an MHC class II-restricted TCR transgene into the CTLA-4$^{-/-}$ mice [228, 229].

There are at least two possible models that can explain why peripheral CD4^{+} T cells appear activated in Jak3- and CTLA-4-deficient mice, based on whether the T cell activation is dependent on the presence of antigen. In the antigen-independent model T cells would become activated because they lack necessary signals that are required to maintain a naïve state. Without those signals, T cells would become activated nonspecifically. In the antigen-dependent model T cell activation would require signals mediated by the TCR in response to a specific self- or environmental-antigen. In this model, only the T cells that receive a TCR signal would become activated. To better understand the mechanisms underlying the T cell activation/expansion and loss of homeostasis in both Jak3$^{-/-}$ and CTLA-4$^{-/-}$ mice, we wanted to investigate whether T cell activation was Ag-dependent or Ag-independent. As mentioned before, both Jak3- and γ_c-deficient mice have been crossed to a variety of MHC class-I- and MHC class-II-dependent TCR transgenic mice. In these models, the mice are not exposed to the specific antigens. For example, Jak3$^{-/-}$ mice crossed to OT-1 TCR transgenic mice do not carry the ovalbumin peptide that would be recognized by the receptor. Since Jak3 deficient TCR transgenic mice do not present the population of activated expanded T cells observed in the straight Jak3 deficient mice, I hypothesized that the activation and expansion of T cells in these mice is dependent on antigen- or specific TCR-mediated activation. In contrast, when mice deficient in CTLA-4 were crossed to MHC class-II

specific TCR transgenic mice, the activation and expansion of the $CD4^+$ T cell population was delayed but not blocked. This observation led us to hypothesize that the activation and expansion of T cells in CTLA-$4^{-/-}$ mice may be independent of antigen-mediated activation.

A.2 Spectratyping; an experimental system to investigate TCR repertoires

One way to investigate whether the $CD4^+$ T cell expansion observed in both mouse models is dependent on the presence of antigen is to analyze the diversity of the peripheral TCR repertoires in each mouse line. If specific antigen recognition is required for the activation and expansion of the peripheral T cells, this would be reflected in the form of a skewed TCR repertoire. Conversely, a diverse and unbiased TCR repertoire *ex vivo*, would indicate that polyclonal T cell activation occurs independent of specific antigen. The TCR repertoire can be analyzed by determining the TCR β-chain gene complexity. The β-chain is composed of the V, D, J, and C regions that are encoded by different gene segments and rearranged to form the final product. The diversity observed between different TCR sequences results not only from the different combinatorial possibilities of V, D, and J gene elements but is additionally enhanced by the removal of bases from the V, D and J segments, as well as the random addition of N nucleotides at the junctions between the V, D and J segments during rearrangement. The VDJ junctional region of the TCR, known as the complementary determining region 3 (CDR3), is the most variable part of the TCR and is thought to be largely responsible for determining the fine peptide specificity and recognition area of the TCR. Due to the

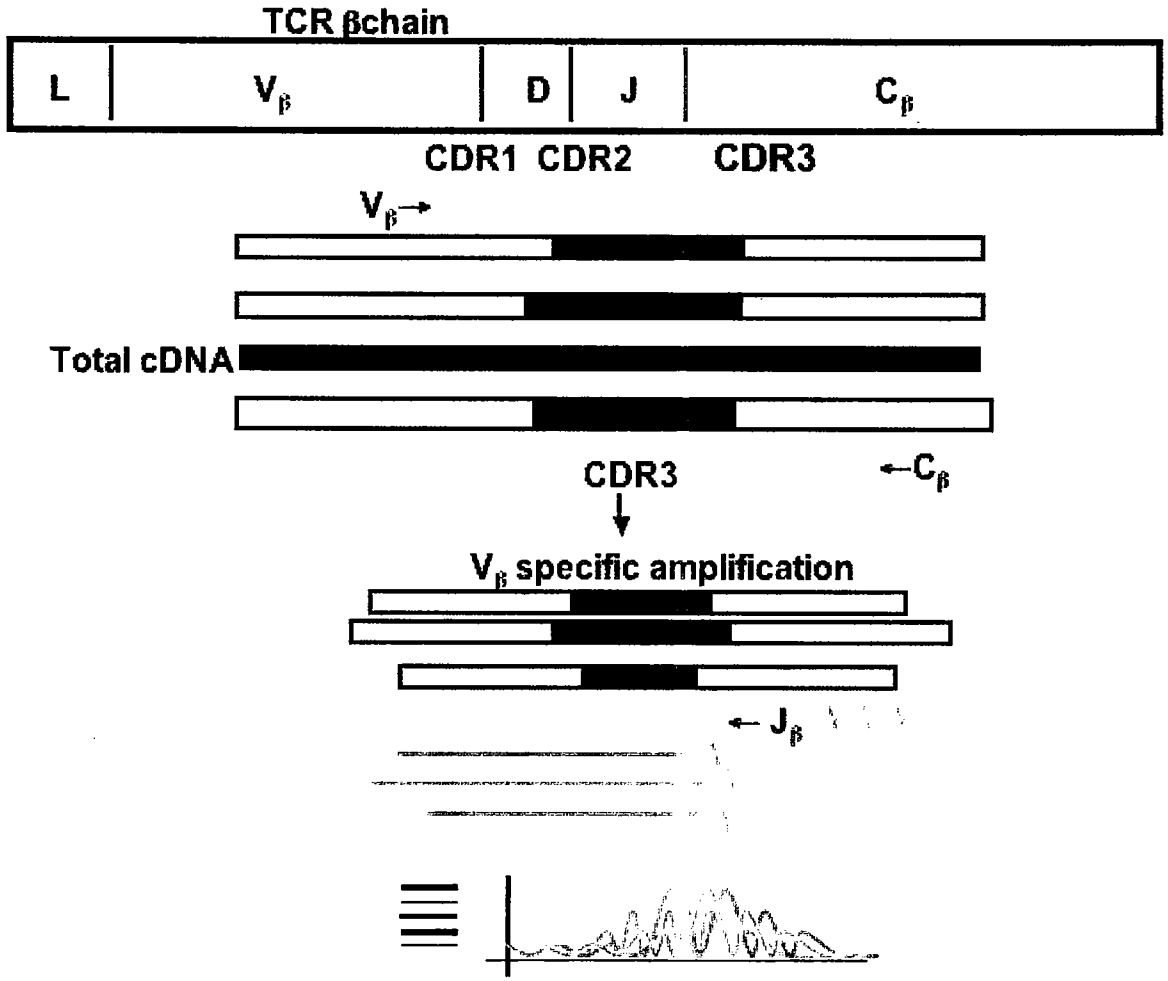
truncation of the gene segments as well as the random DNA elongations that occur during TCR gene recombination, the CDR3 regions of T cells bearing TCR with any give VDJ combination will contain different amino acid sequences and more importantly different lengths. Taking advantage of this particular feature of the TCR gene rearrangement by analyzing the CDR3 length distribution for a specific VDJ combination, provides a powerful way of studying the TCR repertoire diversity [230, 231]. Gorski's group developed a technique called spectratyping which is based on V family specific PCR of the CDR3 region, followed by the resolution of the PCR products by length. A diverse TCR repertoire is indicated by a Gaussian distribution of CDR3 lengths, for a given Vβ-Jβ combination, while a skewed repertoire, such as that seen following antigen specific T cell expansion, is indicated by the preferential accumulation of T cells bearing a CDR3 of one particular length, observed as a single peak, for a given Vβ-Jβ combination (Fig.7) [230, 231].

I demonstrate that the TCR repertoire of the $Jak3^{-/-}$ $CD4^{+}$ T cells is skewed, suggesting that the T cell activation and expansion of peripheral T cells in unmanipulated $Jak3^{-/-}$ mice is antigen-driven. In contrast, the TCR repertoire of activated peripheral T cells from $CTLA4^{-/-}$ mice *ex vivo* remains diverse and unbiased, comparable to that seen in wild type animals. These results demonstrate that the genesis of the T cell expansion in $Jak3^{-/-}$ and $CTLA\text{-}4^{-/-}$ mice is distinct, and suggest a unique role for each of these molecules in the regulation of T cell homeostasis. Further, our results suggest that the repertoire skewing is imposed in the periphery and not during thymic development.

Fig.7 CDR3 length spectratype analysis

Spectratype analysis is a technique based on Vβ specific PCR amplification. It takes advantage of the CDR3 size diversity that results following VDJ recombination. Due to the removal of bases from the V and J segments, as well as the random addition of N nucleotides at the junctions between the V, D and J segments, the size of this region is quite diverse for a given VDJ combination. Total cDNA is synthesized from isolated RNA of a specific organ, such as the spleen. Using a variety of Vβ primers and a constant Cβ primer, the recombined region for each particular Vβ-Cβ combination is amplified. This amplification is followed by PCR run off reactions using fluorescently labeled Jβ primers. The resulting products are run on an acrylimide sequencing gel and the bands are resolved as a series of peaks, each of which represents a given CDR3 length for the specific Vβ-Jβ combination. A diverse TCR repertoire is indicated by a Gaussian distribution of CDR3 lengths, for a given Vβ-Jβ combination, while a skewed repertoire is indicated by the preferential expression of a CDR3, observed as a single peak that represents one particular CDR3 length, for a give Vβ-Jβ combination.

TCR Repertoire (TCR CDR3 length spectratype analysis)



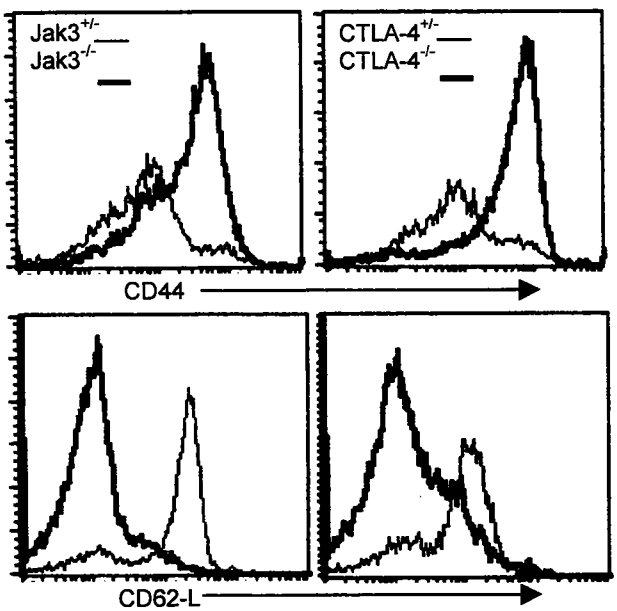
B. RESULTS

B.1 Dysregulation of homeostatic proliferation in Jak3- and CTLA-4 deficient mice

Jak3 and CTLA-4 are two unrelated proteins that function in different pathways during T cell activation; however mice deficient in either of these proteins possess peripheral T cells with a similar phenotype. In both mouse strains, peripheral T cells are large and express high levels of activation markers, such as CD69 and CD44 (Fig.8). They also express high levels of CD25, low levels of CD62-L [179], and a large percentage of them are proliferating *in vivo*, as shown by 5-bromo-2'-deoxyuridine (BrdU) incorporation assays [204, 226]. Additionally, both mouse strains present mostly $CD4^+$ T cells while the percentage and numbers of $CD8^+$ T cells are highly reduced. The nature of the T cell activation is not well understood in either model.

Fig.8 Activated/memory-like T cells populate the periphery of Jak3$^{-/-}$ and CTL-4$^{-/-}$ mice

Splenocytes were stained with Abs to CD4 plus a panel of activation markers. Histograms show the expression of CD44 and CD62L on gated CD4^{+} populations. Thin lines represent heterozygous controls and thick lines represent Jak3$^{-/-}$ (left panels) or CTLA-4$^{-/-}$ (right panels).


Jak3+/-
Jak3-/-
CTLA-4+/-
CTLA-4-/-
CD44
CD62-L

One possibility is that negative selection is defective in these mice, leading to the maturation of self-reactive T cells. However, experimental evidence in both mouse lines suggests that this is an unlikely explanation [142, 226, 232]. Di Santo's group used γ_c deficient mice in the HY system to study the ability of these mice to delete self-reactive $CD8^+$ thymocytes. They found that negative selection of self-reactive $CD8^+$ T cells in the male background was not reduced in the absence of the γ_c chain [11]. Additional experiments were conducted using superantigen-induced deletion as a model for negative selection. The results suggested that the γ_c chain was not required for the superantigen-induced deletion of specific T cells. Finally, using DO10 TCR transgenic mice, Nakajima et al observed that Ag-induced deletion of DP thymocytes took place normally in the absence of the γ_c chain, when the thymocytes were exposed to their specific Ag. but not to a control peptide [203]. Chambers et al analyzed thymocyte development in CTLA-$4^{-/-}$ mice and observed that early thymic differentiation proceeded normally through every checkpoint [226]. Additionally, Waterhouse et al analyzed positive and negative selection in these mice using the HY system, in which TCR^+ thymocytes are negatively selected in male H-2b mice and positively selected in female H-2b mice. When CTLA-4-deficient mice were crossed to the HY TCR transgenic background, positive selection proceeded normally in female mice. The proportions of DP and SP thymocytes were comparable between the CTLA-$4^{-/-}$ and control mice. Similarly, the proportions of DP and SP cells, as well as the numbers of thymocytes were similar between the CTLA-4^+ and CTLA-4^- mice [232]. These results suggest that lack of CTLA-4 does not inhibit positive or negative selection.

There are two alternative possibilities for the dysregulated peripheral T cell activation in Jak3$^{-/-}$ and CTLA-4$^{-/-}$ mice. First, peripheral T cells may expand normally in response to foreign antigens, but have a defect in undergoing activation-induced cell death. A second possibility is that peripheral T cells are being activated by recognition of self-MHC/self-peptide complexes or environmental antigens bound to self-MHC that normally provide survival signals. In order to distinguish between these two possibilities, I analyzed the TCR repertoire of peripheral T cells from Jak3$^{-/-}$ and CTLA-4$^{-/-}$ mice using the CDR3 spectratyping technique [215].

B.2 Mature CD4$^+$ T cells from Jak3-deficient mice present a severely skewed TCR repertoire, while mature CD4$^+$ T cells from CTLA-4 present a normal diverse TCR repertoire

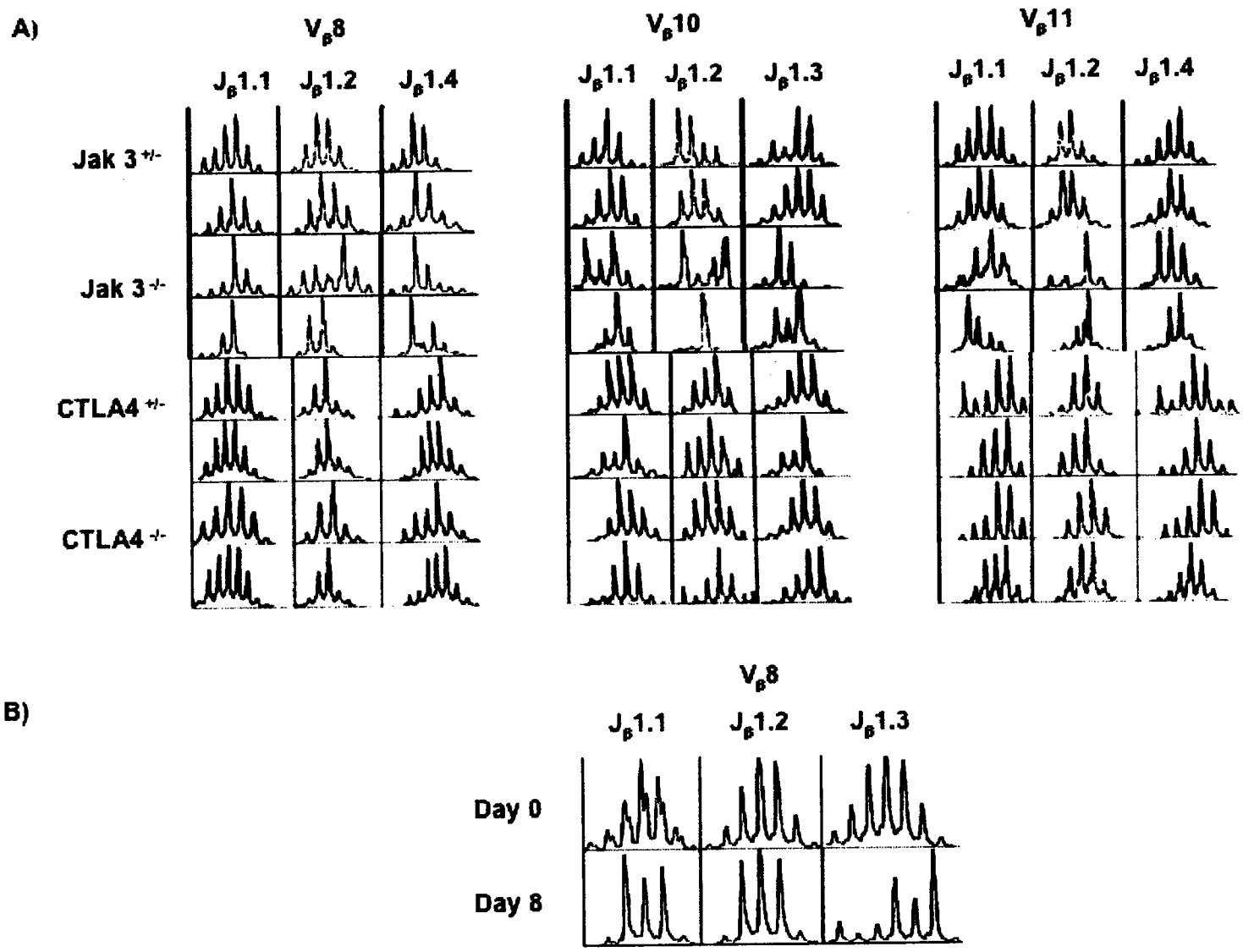
The CDR3 spectratyping technique [215] was chosen because it allows one to examine the length distribution of the most variable part of the TCR, the CDR3 region, which is important in conferring fine peptide specificity of the TCR. Measuring changes in CDR3 length is therefore a more specific means of assessing antigen-mediated changes in T cell repertoire than simply looking at the representation of cells bearing different TCR V_β elements. In addition, CDR3 spectratyping allows for the bulk analysis of the CDR3 region of multiple V_β-J_β combinations, without the need for time-consuming sequencing of individual TCRs. For this technique, total cDNA is amplified by PCR using a C_β-specific primer and individual V_β-primers. The PCR products are then used as templates in elongation reactions using several fluorescently-labeled J_β-specific primers.

The resulting run-off reactions are displayed as a spectrum of size peaks for each CDR3 region. In naïve T cells CDR3 lengths are distributed as a Gaussian curve. An increase in a given peak within the spectrum indicates preferential expansion of a particular T cell clone. As previously established, CDR3 spectratyping provides an exquisitely sensitive means of assessing the heterogeneity of the TCR repertoire in a given population of T cells [215].

To examine the heterogeneity of TCR repertoires in the $Jak3^{-/-}$ and $CTLA\text{-}4^{-/-}$ mice, CDR3 spectratyping was performed on splenocytes using combinations of primers for eight different Vβs and six Jβs (see materials and methods). Representative data is shown in Fig.9 A for three Vβs in combination with three Jβs. Splenocytes from $Jak3^{+/-}$ and $CTLA4^{+/-}$ littermate control mice display a Gaussian distribution, typical of a diverse and unbiased TCR repertoire [29, 215]. This diversity was observed with all combinations of Vβs and Jβs examined (Fig.9 A and data not shown). Interestingly, the same diverse repertoire is observed in splenocytes from $CTLA4^{-/-}$ mice, and was even observed in T cells from $CTLA\text{-}4^{-/-}$ mice with extremely advanced lymphoproliferation Fig.9 A and data not shown). Conversely, CDR3 spectratype analysis of splenocytes from $Jak3^{-/-}$ mice shows a skewed phenotype. The magnitude of skewing appears to be biologically relevant, as it is comparable in magnitude to the skewing observed at the peak of the CTL response to an LCMV infection (day 8), using a primer specific for Vβ8.1 (Fig.9 B and [29]).

Fig.9 Jak3$^{-/-}$ but not CTLA-4$^{-/-}$ peripheral T cells show a skewed TCR repertoire

A) Total RNA was extracted from Jak3$^{+/-}$ and Jak3$^{-/-}$ (8-10 wk of age), CTLA-4$^{+/-}$ and CTLA-4$^{-/-}$ (2 wk of age) splenocytes, and subjected to spectratype analysis as described in *Materials and Methods*. A representative example of the data from two mice of each genotype is displayed for three V_βs, each analyzed with three J_βs. Row 1 (Jak3$^{+/-}$ and CTLA-4$^{+/-}$) and row 4 (Jak3$^{-/-}$ and CTLA-4$^{-/-}$) are littermate controls, while row 2 (Jak3$^{+/-}$ and CTLA-4$^{+/-}$) and row 3 (Jak3$^{-/-}$ and CTLA-4$^{-/-}$) are littermate controls. B) Total RNA was extracted from naive and day 8 LCMV-infected C57BL/6 splenocytes and subjected to spectratype analysis using a V8.1-specific in combination with different J_β specific primers.


A)
Vβ8
Vβ10
Vβ11
Jβ1.1
Jβ1.2
Jβ1.4
Jβ1.3
Jak 3 +/-
Jak 3 -/-
CTLA4 +/-
CTLA4 -/-
B)
Vβ8
Jβ1.1
Jβ1.2
Jβ1.3
Day 0
Day 8

To confirm this initial observation, these experiments were repeated with a total of five Jak3$^{+/-}$ mice, seven Jak3$^{-/-}$ mice, four CTLA-4$^{+/-}$ mice and four CTLA-4$^{-/-}$ mice, each analyzed with four Vβ primers coupled with six Jβ primers. Of these, three of the Jak3$^{+/-}$ and five of the Jak3$^{-/-}$ mice were analyzed with primers specific for an additional four Vβ's in combination with six Jβs. In all cases, the data supported the initial observation that peripheral T cells from Jak3$^{-/-}$ mice have a profoundly skewed TCR repertoire, whereas peripheral T cells from the other three groups tested, including the CTLA-4$^{-/-}$ mice exhibited a normal, diverse and unbiased repertoire (data not shown). These data strongly support the idea of antigen-dependent activation and expansion of Jak3$^{-/-}$ T cells, as only a limited number of T cell clones appears to be expanded in these mice. In contrast, the activation and expansion of CTLA-4$^{-/-}$ T cells appears to occur by an antigen-independent mechanism, as an unlimited number of T cell clones is expanding in the absence of CTLA-4. Interestingly, skewing of the CDR3 region was detected in the Jak3$^{-/-}$ T cells despite the fact that there was no detectable skewing of the TCR repertoire as assessed by Vβ and Vα usage determined by flow cytometry [233].

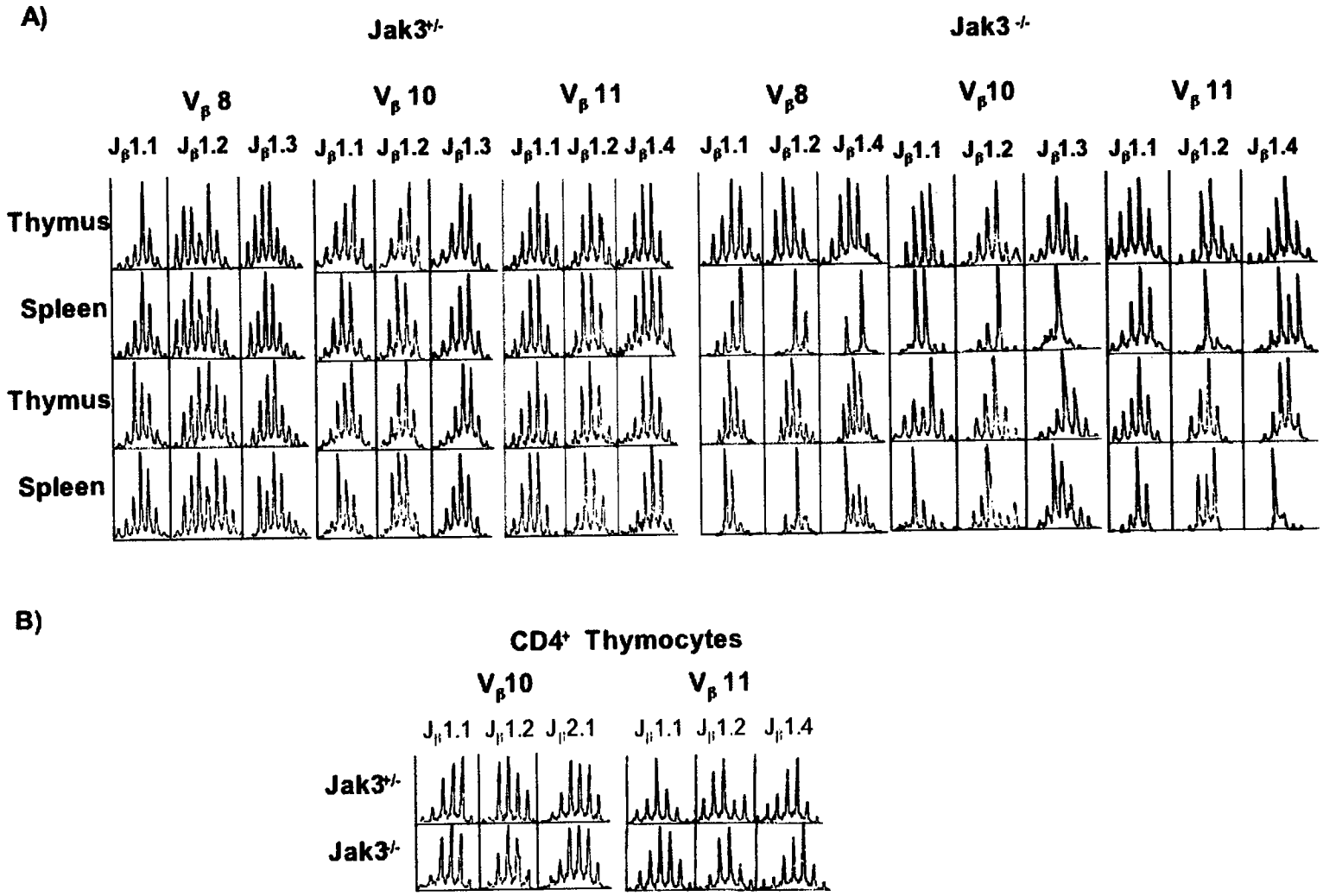
B.3 In contrast to peripheral mature T cells, immature CD4^{+} T cells present a diverse TCR repertoire in the thymus of Jak3-deficient mice

To address the possibility that skewing of the TCR repertoire in Jak3$^{-/-}$ mice is occurring during thymic selection, rather than as a result of peripheral T cell activation and expansion, I examined the TCR repertoire of Jak3$^{-/-}$ thymocytes. CDR3 spectratype analysis was performed using total thymocytes from Jak3$^{-/-}$ and Jak3$^{+/-}$ mice. As can be

seen in Fig.10 A, thymocytes from $Jak3^{-/-}$ mice show the typical Gaussian distribution of a diverse TCR repertoire, whereas splenocytes show a highly skewed repertoire. To further confirm that the skewing observed in the peripheral T cell compartment was not imposed during negative selection, and to eliminate the potential contribution of unselected DP thymocytes, I also examined the TCR repertoire of purified $CD4^+$ single positive thymocytes from $Jak3^{-/-}$ and control ($Jak3^{+/-}$) mice. Both the $Jak3^{+/-}$ and the $Jak3^{-/-}$ $CD4^+$ single positive thymocytes exhibited a diverse and unbiased TCR repertoire (Fig.10 A and 3 B), demonstrating that the skewing observed in the TCR repertoire of peripheral $Jak3^{-/-}$ T cells does not occur as a result of altered positive or negative selection in the thymus.

Fig.10 The TCR repertoire skewing in Jak3$^{-/-}$ mice occurs in the periphery, not in the thymus

A) Total RNA was isolated from Jak3$^{+/-}$ and Jak3$^{-/-}$ (8–9 wk of age) thymi and spleens and subjected to spectratype analysis. A representative example of the data for two mice of each genotype is displayed for three V_βs, each analyzed with three J_βs. The first Jak3$^{+/-}$ and the second Jak3$^{-/-}$ are littermate controls while the second Jak3$^{+/-}$ and the first Jak3$^{-/-}$ are littermate controls. B) Total RNA was extracted from sorted CD4$^+$ thymocytes from Jak3$^{+/-}$ and Jak3$^{-/-}$ thymi and subjected to spectratype analysis. A representative example of the data for two V_βs, each analyzed with three J_βs, is shown.


A)
Jak3+/-
Jak3 -/-
Vβ 8
Vβ 10
Vβ 11
Vβ8
Vβ10
Vβ 11
Jβ1.1 Jβ1.2 Jβ1.3 Jβ1.1 Jβ1.2 Jβ1.3 Jβ1.1 Jβ1.2 Jβ1.4 Jβ1.1 Jβ1.2 Jβ1.4 Jβ1.1 Jβ1.2 Jβ1.3 Jβ1.1 Jβ1.2 Jβ1.4
Thymus
Spleen
Thymus
Spleen
B)
CD4+ Thymocytes
Vβ10
Vβ 11
Jβ1.1 Jβ1.2 Jβ2.1 Jβ1.1 Jβ1.2 Jβ1.4
Jak3+/-
Jak3-/-

C. DISCUSSION

Deficiencies in both Jak3 and CTLA-4 lead to a profound dysregulation in T cell homeostasis. Jak3-deficient and CTLA-4-deficient mice are characterized by the development of peripheral activated/memory-like $CD4^+$ T cells. In both cases, the majority of mature T cells are $CD4^+$ and have upregulated the activation marker CD44. Previous studies have suggested that thymocyte development, as well as positive and negative selection take place relatively normally in both models; therefore, the defect in T cell homeostasis is likely to take place following entry into the periphery. Despite these similarities, the data presented suggest that the defect in T cell homeostasis and the development of activated/memory-like $CD4^+$ T cells observed in the periphery of $Jak3^{-/-}$ and CTLA-$4^{-/-}$ mice are produced by different mechanisms. The sprectatyping analysis clearly shows that the $CD4^+$ T cells in the periphery of mice deficient in Jak3 have a restricted TCR repertoire. The skewed TCR repertoire is not apparent in the thymus of $Jak3^{-/-}$ mice, suggesting that the skewing takes place after the T cells enter the periphery. These observations indicate that only a limited number of T cell clones proliferate and populate the periphery of $Jak3^{-/-}$ mice, suggestive of an Ag-mediated activation of these cells. Contrary to these results, the spectratyping analysis reveals that the $CD4^+$ T cells in mice deficient in CTLA-4 present a normal, diverse repertoire, indicating that a polyclonal population fills the periphery of CTLA-$4^{-/-}$ mice. These observations suggest that $CD4^+$ T cells become activated nonspecifically, in an Ag-independent fashion.

These results, together with our previous studies of $Jak3^{-/-}$ TCR transgenic mice, in which I saw that T cells do not proliferate or become activated in the absence of the

specific Ag, suggest the following model. Jak3$^{-/-}$ αβTCR^{+} thymocytes undergo normal positive and negative selection in the thymus and migrate to the periphery as resting naïve T cells. Once in the periphery, naïve Jak3$^{-/-}$ T cells are susceptible to apoptosis, due to the lack of IL-7 signaling in the absence of Jak3. However, other mechanisms may also play a role. A fraction of newly developed Jak3$^{-/-}$ T cells encounter specific antigens in the periphery, and these cells get activated and expand. Once activated, the Jak3$^{-/-}$ CD4^{+} T cells will become independent of IL-7 for survival, and will accumulate in the periphery. This accumulation may be due to the absence of IL-2R-mediated upregulation of FasL, necessary for activation-induced cell death. Consistent with this hypothesis, Nakajima et al have previously shown that γ_c signaling is essential for the deletion of activated peripheral CD4^{+} T cells, most likely by inducing FasL expression [142, 234]. Alternatively, the accumulation of CD4^{+} CD44^{+} T cells may be due to homeostatic proliferation of these cells. Unlike naïve CD4^{+} T cells, memory CD4^{+} CD44^{+} T cells do not require γ_c cytokines to survive or homeostatically proliferate in a T cell depleted environment. Since the thymus cellularity of Jak3-deficient mice is greatly reduced, the numbers of T cells entering the periphery are highly reduced, compared to normal mice. Therefore, activated/memory CD4^{+} T cells may have the space and/or necessary factors required to proliferate and repopulate the periphery. In contrast to memory CD4^{+} T cells, both naïve and memory CD8^{+} T cells require γ_c cytokines to survive and homeostatically proliferate. Several reports have demonstrated that naïve CD4^{+} and CD8^{+} T cells cannot survive in the absence of IL-7 signals, while CD8^{+} memory T cells require IL-15 and to a lesser extent IL-7 signals. The same requirements

appear to be necessary for homeostatic proliferation of these cells *in vivo*. It is not clear what cytokines or factors contribute to the survival and proliferation of memory $CD4^+$ T cells, but it is clear that γ_c cytokines are not required. Therefore, in the absence of Jak3/γ_c signals, only $CD4^+$ T cells that receive a TCR signal and become memory-like are able to survive and proliferate as mature T cells.

Similar to the Jak3-deficient mice, CTLA-$4^{-/-}$ animals do not appear to have defects in $\alpha\beta TCR^+$ thymocyte selection, and single positive thymocytes emigrate to the periphery as mature naïve T cells [226]. However, almost immediately upon entering the periphery, CTLA-$4^{-/-}$ T cells become activated [226]. The subsequent accumulation of these T cells in the periphery does not appear to be due to a defect in apoptosis [227, 235]. Instead, we have proposed that CTLA-$4^{-/-}$ T cells can become activated as a result of some of the TCR/MHC interactions necessary for peripheral T cell survival/homeostasis (18, 27, 28). Further, as CTLA-4-mediated inhibition is more profound in previously activated T cells, compared to naïve T cells [101, 228], the absence of CTLA-4 would be magnified upon re-stimulation of the CTLA-$4^{-/-}$ T cells *in vivo*. This model predicts that exogenous antigens would not be necessary for activation of CTLA-$4^{-/-}$ T cells, and that there should be no skewing of the TCR repertoire. The results presented here support this model. Also consistent with this model is the observation that $CD4^+$ T cells become activated in H-2M$\alpha^{-/-}$ mice in the absence of CTLA-4 (Chambers CA, unpublished observation).

In summary, these CDR3 spectratype results demonstrate that the expansion of T cells observed in Jak$3^{-/-}$ mice does not occur during thymic development, but instead,

takes place in the periphery of the mice and involves a restricted number of T cell clones. In contrast, the expansion of T cells in the periphery of CTLA4$^{-/-}$ mice does not appear to be restricted to a limited number of T cell clones, as the diversity of the TCR repertoire in CTLA-4$^{-/-}$ mice is comparable to unimmunized wild type mice. Therefore, two very similar phenotypes of peripheral T cell activation and expansion are clearly derived by distinct mechanisms.

CHAPTER IV

T CELL DEFECTS IN JAK-3-DEFICIENT MICE ARE NOT RESCUED BY THE ENFORCED EXPRESSION OF BCL-2

A. INTRODUCTION

Adaptive immunity is dependent on the proper function of mature lymphocytes. This requires a balance between the host response to foreign antigens and unresponsiveness to self-antigens. T cells undergo a process of development by which they acquire unique surface receptors that determine not only which lymphocytes successfully mature, but also the ability of mature lymphocytes to recognize foreign antigens and mount appropriate immune responses [12]. Proper T cell development is therefore crucial for the function of the immune system. The thymus provides a unique combination of cellular interactions, cytokines, and chemokines that mediate the proliferation and differentiation of thymocytes into functional T cells [44, 236]. The first thymocyte precursors are termed triple negative cells ($CD3^-CD4^-CD8^-$) and are subdivided into TN1-TN4 based on their expression of CD44 and CD25 [8-10]. The first precursor group, TN1, is classified as $CD44^+CD25^-$c-kit^+IL-$7R\alpha^-$ and it depends on many cytokines for survival [20, 237]. The next group, TN2, is characterized by an upregulation of the IL-7Rα chain, CD25 and the pre-TCRα chain. These cells also undergo substantial expansion, mediated by the tyrosine kinase receptor c-kit and the γ_c chain-dependent cytokine IL-7. The thymi of c-kit/γ_c double mutant mice, but not c-kit or γ_c single mutants are alymphoid, and TCR gene rearrangements in these thymi are undetectable [238]. In the following stage, TN3, expression of CD44 and c-kit, as well as IL-7Rα are downregulated, and the cells begin to rearrange their TCR genes. At this point the cells commit to the T cell lineage [20, 239]. The Notch-1 receptor is essential for this commitment [240]. Rearrangement of the β chain leads to its association with the

pre-Tα chain and CD3 subunits, and subsequently to the surface expression of the pre-TCR [21, 241, 242]. Signaling through this receptor triggers the differentiation of thymocytes into double positive ($CD4^+$ $CD8^+$) cells [243, 244]. During this stage the TCR-α chain is rearranged and expression of the mature CD3-TCRαβ complex is first detected. Thymocytes then undergo positive and negative selection, which result in the survival and differentiation of thymocytes that can recognize peptide in the context of self-MHC but do not have too strong an avidity for self-peptide-self-MHC complexes [19, 245].

A.1 The γ_c cytokine IL-7 plays a non-redundant role in thymocyte development

The γ_c chain family of cytokines is extremely important for the development, maintenance and function of murine lymphocytes [43, 47-49, 224, 238]. Surprisingly, although IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 share the γ_c chain, IL-7 plays a non-redundant role in thymocyte development [185]. The lymphopenia observed in γ_c chain- and Jak3-deficient mice, is very similar to that observed in IL-7R-deficient mice [43, 49, 189]. The thymi of the three mouse models are extremely reduced in cellularity, about 1-10% that of a normal thymus. Development of γδ T cells is completely blocked, whereas, despite very reduced numbers, development of αβ T cells seems relatively normal in the absence of γ_c, Jak3 or IL-7R signaling. One major difference observed between the Jak3/γ_c-deficient and the IL-7R-decifient mice is the absence of NK cells in the former models [193, 194]. IL-15, also a member of the γ_c chain family, is essential

for the development and survival of NK cells. Signaling through this cytokine is unaffected in IL-7R$^{-/-}$ mice.

A.2 IL-7 induced Bcl-2 expression mediates the survival of specific thymocyte populations during T cell development

The antiapoptotic protein Bcl-2 also plays an essential role during T cell development in the thymus. Bcl-2 inhibits some, but not all forms of stimuli that lead to apoptosis [246]. Its expression is tightly regulated in thymocytes throughout development; it is upregulated in TN thymocytes, downregulated in DP thymocytes and upregulated again, following selection, in mature naïve T cells [15, 17]. Thymocyte development is initially normal in Bcl-2 deficient mice, but about 6 weeks of age all thymocytes and peripheral T cells in these mice become severely sensitive to apoptotic signals. Since deficiencies in Bcl-2 result in the death of the mice a few weeks after birth, a detailed analysis of lymphopoiesis in the absence of Bcl-2 has been very difficult [14]. However, clear similarities between Bcl-2-deficient and IL-7-deficient mice, in terms of defects during lymphoid development, have been described [247]. In order to better understand the relationship between Bcl-2 and IL-7, Von Freeden-Jeffry et al analyzed TN cells from IL-7$^{-/-}$ mice by looking at the expression of Bcl-2, levels of apoptosis and cell cycle progression [187]. They observed that crossing IL-7 knock-out mice to RAG knock-out mice led to the development of thymi almost completely devoid of thymocytes, indicating that IL-7 signaling was needed for the survival of thymocytes prior to TCR rearrangement. They also observed that IL-7 signaling was necessary for

expression of Bcl-2 in the TN2, TN3 and TN4 populations, and that a decrease in the expression of Bcl-2 resulted in increased apoptosis of these cell subsets. Additionally, IL-7-deficient TN thymocytes showed significantly fewer cells undergoing cell-cycle progression. Finally, when IL-7-deficient thymocytes were incubated *in vitro* in the presence of recombinant IL-7, Bcl-2 expression was induced, and the cells were 95% more viable than in the absence of the cytokine [187].

A.3 Effect of the enforced expression of Bcl-2 during T cell development in the absence of IL-7- and γ_c-mediated signals

Given these results, several groups went on to investigate whether constitutive expression of Bcl-2 could rescue the defects observed in thymocyte development in IL-7- and γ_c-deficient mice. Irving Weissman's group conducted experiments in which thymocytes from MHC-deficient mice were adoptively transferred into the thymi of WT $MHC^{+/+}$ mice, either in the presence or absence of neutralizing IL-7Rα Ab (A7R34) [248]. They observed that, in the presence of A7R34, neither DN nor DP thymocytes were able to undergo positive selection and become SP cells, whereas both DN and DP thymocytes underwent positive selection and became SP cells in the absence of the neutralizing Ab. However, when they injected thymocytes from $MHC^{-/-}$ Eμ-bcl-2 transgenic mice (these mice express human Bcl-2 in cells of the T and B cell lineage, under the Eμ promoter), into $MHC^{+/+}$ thymi, they observed that A7R34 did not block their differentiation into SP cells. Therefore, they concluded that IL-7-mediated signals are necessary to maintain the viability of DN and DP thymocytes, in order for them to

differentiate into mature T cells [248]. Furthermore, by crossing IL-7Rα$^{-/-}$ mice to Eμ-bcl-2 transgenic mice they were able to partially restore T cell development. The Eμ-bcl-2 IL-7Rα$^{-/-}$ thymus exhibited a 10-fold increase in the number of thymocytes compared to that seen in IL-7Rα$^{-/-}$ mice, and the CD4 to CD8 ratio of SP thymocytes was comparable to that of a normal thymus. The expression of Bcl-2 significantly restored the generation of all maturational stages of development. Additionally, there was a 7-fold increase in the numbers of T cells seen in the blood and an 11-fold increase in T cell numbers in the spleen of these mice, resulting in a partial restoration of peripheral T cell numbers. Finally, they observed that mature T cells from the IL-7Rα$^{-/-}$ Eμ-bcl-2 mice responded significantly better to concanavalin A or CD3 plus CD28 stimulation than IL-7Rα$^{-/-}$ T cells, and almost as well as WT cells, though there was variability in these results. Maraskovsky et al observed even more striking results when they used the same system. By crossing the IL-7Rα$^{-/-}$ mice to the Eμ-bcl-2 transgenic mice, they rescued thymocyte numbers to normal developmental levels [249].

Weissman's group also crossed $\gamma_c^{-/-}$ mice to Eμ-bcl-2 transgenic mice and to transgenic mice that express Bcl-2 under the H-2K promoter [18]. Whereas expression of the Eμ-bcl-2 transgene was observed in 85% of CD3$^+$ T cells, the expression of H-2K-bcl-2 was found in all blood leukocytes. In these studies, they observed that the absolute number of thymocytes, as well as the number of thymocytes that have recently received positive selection signals (CD69$^+$), were increased in both $\gamma_c^{-/-}$ Eμ-bcl-2 transgenic mice (20% of WT levels) and $\gamma_c^{-/-}$ H-2K-bcl-2 mice (50% of WT levels). The investigators concluded that the more efficient restoration observed in the H-2K-bcl-2 transgenic mice

may have been due to the fact that the H-2K promoter is expressed in virtually all thymocytes whereas the Eμ-bcl-2 transgene is not expressed in early thymic precursors ($CD3^-CD25^-c\text{-}kit^+$).

In contrast to these results, Rodewald et al observed that the Eμ-bcl-2 transgene was expressed in the $CD3^-CD25^-c\text{-}kit^+$ precursors and further, that it protected these cells from growth factor withdrawal-induced apoptosis *in vitro* [250]. When they analyzed the thymus cellularity of adult γ_c-deficient and γ_c-deficient Eμ-bcl-2 transgenic mice, they observed that there was a wide range of cell numbers in both mouse models that spread over two logs. Furthermore, when comparing γ_c^- and γ_c^- bcl-2^+ mice, they concluded that neither the range, nor the mean values of thymocytes numbers were significantly different between the two groups, and both were considerably reduced compared to WT mice. Rodewald and colleagues went on to analyze the distribution of TN thymocytes in WT, γ_c-deficient, and γ_c-deficient Eμ-bcl-2 transgenic mice, to determine whether the developmental block observed in γ_c-deficient mice (arrested at the transition from the TN2 ($CD44^+CD25^+$) to the TN3 ($CD44^-CD25^+$) stage) had been reversed in the presence of Bcl-2, without affecting the overall thymocyte numbers. While the distribution of TN precursors in WT mice was normal (11% TN2, 63% TN3, and 21% TN4), the distribution of TN precursors in $\gamma_c^{-/-}$bcl-2^+ displayed the same block observed in γ_c-deficient mice (60% TN2, 37% TN3, and 1% TN4). They argued that if the developmental block observed in the $\gamma_c^{-/-}$deficient mice had been rescued, the percentage of TN2 would have decreased while the percentage of TN3 and, most importantly, TN4

would have increased. They concluded that enforced expression of Bcl-2 does not rescue thymocyte development in the absence of γ_c chain signaling [250].

Finally, Leonard's group investigated the effect of enforced expression of Bcl-2 in γ_c-deficient mice, with or without a transgenic TCR [17]. They used a Bcl-2 transgene driven by the Lck promoter and saw that in these transgenic mice over 99% of the thymocytes expressed Bcl-2. When these mice were crossed to the $\gamma_c^{-/-}$ mice, the total numbers of thymocytes were increased about 4-fold compared to the non-transgenic $\gamma_c^{-/-}$ mice, but the thymic cellularity was still only 12% of that in WT mice. The percentage of $CD8^+$ thymocytes also increased in $\gamma_c^{-/-}$bcl-2^+ transgenic mice, normalizing the CD4 to CD8 ratio. However, the presence of Bcl-2 did not rescue the defect in cell cycle progression observed in $\gamma_c^{-/-}$ thymocytes. Next, they investigated the thymic and splenic cellularity of γ_c-deficient mice crossed to DO 10 TCR transgenic mice in different MHC backgrounds. The DO10 TCR exhibits higher affinity for I-A^b than I-A^d and more thymocytes are usually detected in H-$2^{d/d}$ mice than in H-$2^{d/b}$ mice, due to negative selection in the H-$2^{d/b}$ background. This was also true in the absence of the γ_c chain. Although there was an increase in the total numbers of thymocytes upon crossing the $\gamma_c^{-/-}$ mice to the $DO10^+$ mice in the H-$2^{d/d}$ background, the numbers were still reduced compared to the γ_c^+ $DO10^+$ control mice. Additionally, the total numbers of splenocytes in the $\gamma_c^{-/-}$ $DO10^+$ H-$2^{d/d}$, and especially in the H-$2^{d/b}$ mice were highly reduced compared to the non transgenic γ_c-deficient mice. When they investigated thymocyte development in $\gamma_c^{-/-}$ $DO10^+$ H-$2^{d/b}$ mice, in the presence of Bcl-2, they observed that Bcl-2 increased the number of thymocytes compared to $DO10^+$ γ_c-deficient mice, but the numbers were

still highly reduced compared to WT DO10$^+$ mice. Bcl-2 expression also increased the percentage and numbers of splenic DO10 TCR transgenic cells in $\gamma_c^{-/-}$ mice. This increase in thymocyte and splenocyte numbers was noticeable in the H-2$^{d/b}$ background, but not in the H-2$^{d/d}$ background. Leonard's group concluded that the importance of Bcl-2 may be dependent on the TCR-MHC affinity [17].

A.4 From the thymus to the periphery; the role of the γ_c chain in T cell homeostasis

Following development in the thymus, mature T cells enter the periphery where they circulate from the blood to the secondary lymphoid organs, surveying the body for infecting pathogens [52]. Unless they encounter their specific antigen, mature T cells survive in the periphery for a limited period of time, as naïve cells [54-56]. The numbers of peripheral T cells, the ratio of naïve, activated and memory cells, as well as the ratio between CD4$^+$ and CD8$^+$ T cells are tightly controlled to achieve a desirable state of homeostasis, which is crucial for the immune system [57, 59, 66, 67, 251]. The regulation of T cell homeostasis is dependent on signals mediated by cell-surface molecules and soluble molecules.

The absence of γ_c cytokine signaling leads to a dramatic dysregulation in T cell homeostasis [73-75, 252]. Specifically, the small thymi in Jak3-deficient and γ_c-deficient mice produce reduced numbers of peripheral T cells; however, peripheral T cell numbers in these mice achieve normal levels by three weeks of age [179-181]. The vast majority of these peripheral T cells are CD4$^+$ and present an activated or memory phenotype, characterized by the increased expression of CD44 and the downregulation of CD62L.

IL-$7^{-/-}$ and IL-$7R^{-/-}$ mice have reduced numbers of peripheral T cells, confirming the important role played by this cytokine in T cell homeostasis [56, 57]. Furthermore, the levels of Bcl-2 in mature $CD44^{lo}$ $CD4^+$ and $CD8^+$ T cells from γ_c- and Jak3-deficient mice are highly reduced [187], indicating that expression of Bcl-2 is mediated, to an extent, by γ_c-dependent signals. As discussed above, whether or not enforced expression of Bcl-2 can rescue mature naïve $CD4^+$ and $CD8^+$ T cells, in the absence of γ_c/Jak3-mediated signals, appears to be controversial.

Due to the conflicting results and the possibility that Bcl-2 expression may rescue a population of naïve T cells that lack Jak3/γ_c-mediated signals, I decided to cross the Jak3-deficient mice to the Eμ-bcl-2 transgenic mice. I investigated thymocyte development, as well as peripheral homeostasis in $Jak3^{-/-}$bcl-2 transgenic mice. I did not observe a significant rescue in thymocyte development or naïve T cell survival. Additionally, I crossed the $Jak3^{-/-}$bcl-2 transgenic mice to the OT-1 transgenic mice and detected the rescue of a naïve $CD8^+$ $OT\text{-}1^+$ T cell population in the spleens of these mice. The results are discussed below.

B. RESULTS

B.1 Enforced expression of Bcl-2 does not rescue the developmental defect observed in Jak3-deficient thymi

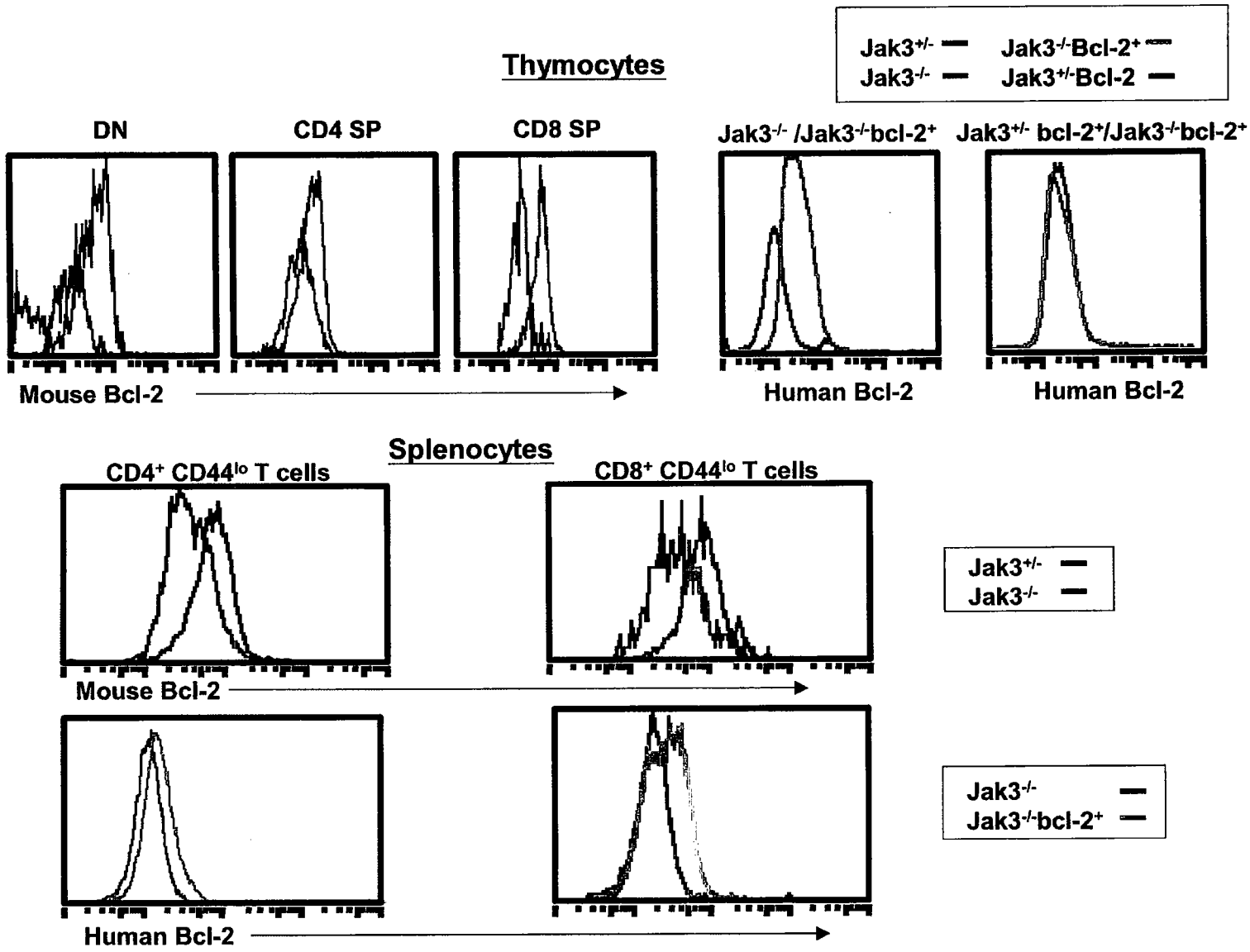
I crossed $Jak3^{-/-}$ mice to Eμ-bcl-2 transgenic mice to obtain $Jak3^{-/-}$bcl-2^+ and $Jak3^{+/-}$bcl-2^+ control mice. Upon dissecting the mice, I immediately noticed that the thymi of $Jak3^{-/-}$bcl-2^+ mice were extremely reduced in size, compared to the $Jak3^{+/-}$ or the

$Jak3^{+/-}bcl\text{-}2^{+}$ control thymi. In fact, the size of the thymi was not much bigger than that of nontransgenic $Jak3^{-/-}$ mice. Furthermore, $Jak3^{-/-}bcl\text{-}2^{+}$ mice, like $Jak3^{-/-}$ and unlike $Jak3^{+/-}$ mice, did not have visible lymph nodes. The size of the spleen, as has previously been observed in $Jak3^{-/-}$ mice, was highly variable between individual mice.

I analyzed the expression of Bcl-2 in total thymocytes, $CD4^{+}$ and $CD8^{+}$ mature T cells. As previously reported, $Jak3^{-/-}$ mice presented reduced levels of endogenous Bcl-2, both in thymocytes and naïve mature peripheral T cells compared to $Jak3^{+/-}$ control littermates (Fig.11). Both thymocytes and mature T cells from $Jak3^{-/-}bcl\text{-}2^{+}$ mice had higher levels of human Bcl-2 expression compared to $Jak3^{-/-}$ littermates. The levels of human Bcl-2 expression observed in $Jak3^{+/-}bcl\text{-}2^{+}$ and $Jak3^{-/-}bcl\text{-}2^{+}$ mice were comparable (Fig.11).

Fig.11 Expression of Bcl-2 in $Jak3^{-/-}$ and $Jak3^{-/-}$bcl-2 transgenic mice

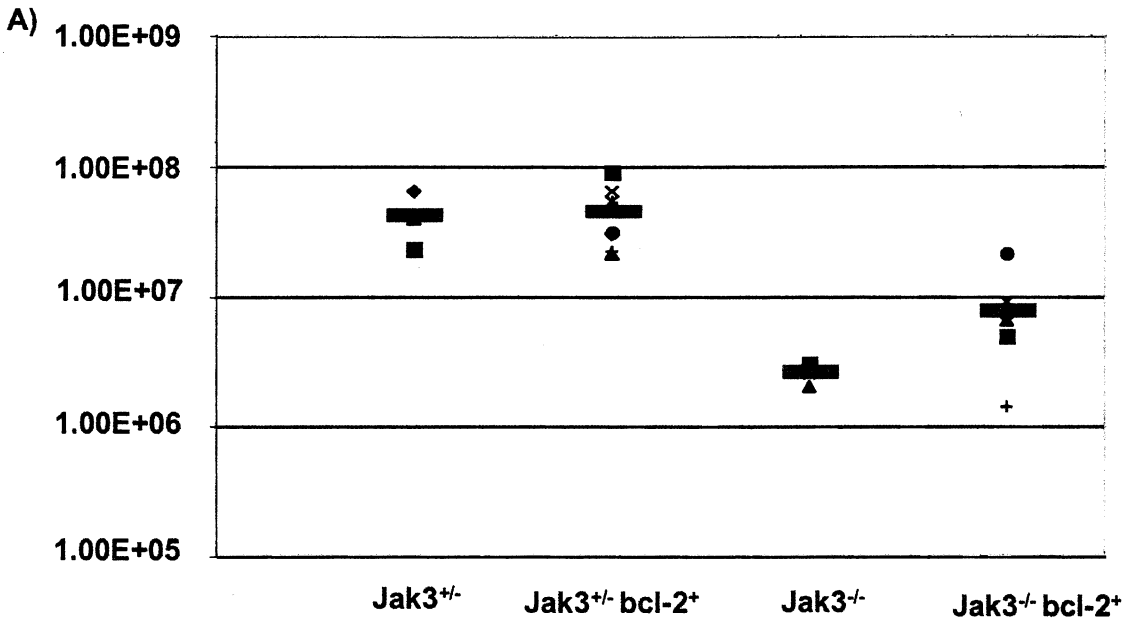
Single cell suspensions from whole thymi and whole spleens were stained with CD4, CD8 and CD44 mAbs for surface expression of the molecules, fixed and permeabilized for intracellular staining of either mouse or human Bcl-2. The first three to panel depict the expression of mouse Bcl-2 by different thymocyte populations from $Jak3^{-/-}$ and $Jak3^{+/-}$ mice, the fourth top panel depicts expression of human Bcl-2 from $Jak3^{-/-}$ and $Jak3^{-/-}$bcl-2^{+} mice, and the fifth top panel depicts expression of human Bcl-2 between $Jak3^{-/-}$bcl-2^{+} and $Jak3^{+/-}$bcl-2^{+} mice. The middle row of panels depicts the expression of endogenous mouse Bcl-2 from $Jak3^{-/-}$ and $Jak3^{+/-}$ mice. The bottom row of panels depicts the expression of human Bcl-2 from $Jak3^{-/-}$ and $Jak3^{-/-}$bcl-2^{+} mice.

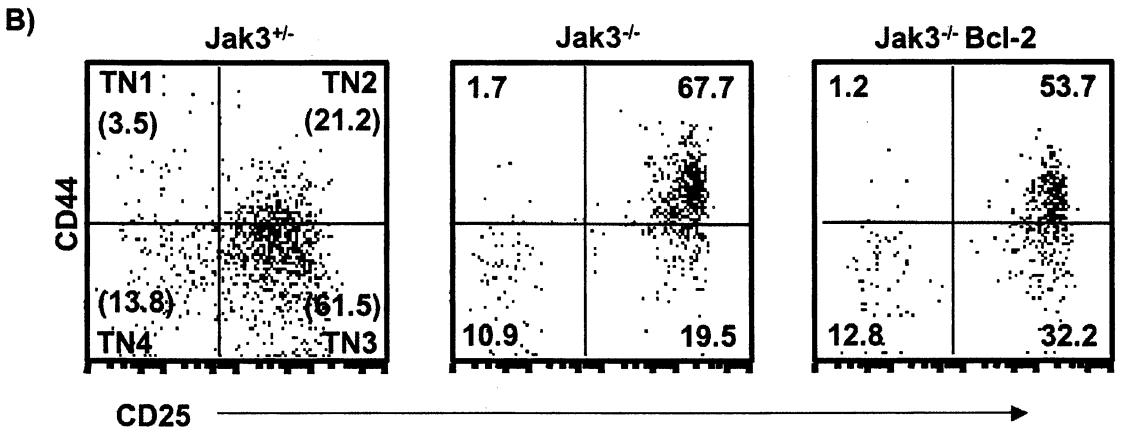

Thymocytes
Jak3+/-
Jak3-/-
Jak3-/-Bcl-2+
Jak3+/-Bcl-2
DN
CD4 SP
CD8 SP
Jak3-/- /Jak3-/-bcl-2+
Jak3+/- bcl-2+/Jak3-/-bcl-2+
Mouse Bcl-2
Human Bcl-2
Human Bcl-2
Splenocytes
CD4+ CD44lo T cells
CD8+ CD44lo T cells
Jak3+/-
Jak3-/-
Mouse Bcl-2
Jak3-/-
Jak3-/-bcl-2+
Human Bcl-2

As mentioned above, the size of the thymus was still very small in $Jak3^{-/-}$ mice that expressed Bcl-2. There was a significant increase in the total numbers of thymocytes compared to the nontransgenic Jak3-deficient mice, but the levels were still reduced compared to the heterozygous mice (Fig.12 A). A detailed analysis of thymocyte development, examining the expression of CD44 and CD25 in lineage marker (CD3, CD4, CD8, B220, Ter119, Mac 1, Gr-1, DX5)-negative thymocytes, revealed that Bcl-2 expression did not rescue the $Jak3^{-/-}$ developmental defect. As previously described, $Jak3^{-/-}$ mice presented a developmental block between the TN2 ($CD44^{+}CD25^{+}$) and the TN3 ($CD44^{-}CD25^{+}$) stages, and had very few TN4 ($CD44^{-}CD25^{-}$) thymocytes compared to $Jak3^{+/-}$ mice (Fig.12 B). The same was true for $Jak3^{-/-}$bcl-2^{+} mice. The percentage of TN2 thymocytes was elevated compared to $Jak3^{+/-}$ mice, while the percentages of TN3 and especially TN4 thymocytes appeared reduced (Fig.12 B). These results concur with the observations reported by Rodewald et al upon investigation of TN thymocyte development in $\gamma_c^{-/-}$bcl-2^{+} transgenic mice.

Fig.12 Enforced expression of Bcl-2 leads to increased thymocyte numbers but does not rescue the developmental defect in Jak3$^{-/-}$ mice

Single cell suspensions of thymi from Jak3$^{+/-}$, Jak3$^{-/-}$, Jak3$^{+/-}$bcl-2^{+}, and Jak3$^{-/-}$bcl-2^{+} mice were prepared, A) total thymocytes counted and stained with Abs for CD4 and CD8. The red lines represent the mean value of each sample. B) Populations of TN thymocytes were investigated by gating out other cell populations through staining with CD3, CD4, CD8, B220, Ter119, Mac 1, Gr-1, DX5 Abs and examining CD25 and CD44 expression on the negative population. The numbers in each quadrant represent the percentage of each cell population within the TN population.


A)
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
Jak3+/-
Jak3+/- bcl-2+
Jak3-/-
Jak3-/- bcl-2+


B)
Jak3+/-
Jak3-/-
Jak3-/- Bcl-2
TN1
(3.5)
TN2
(21.2)
(13.8)
TN4
(61.5)
TN3
1.7
67.7
10.9
19.5
1.2
53.7
12.8
32.2
CD44
CD25

B.2 Enforced expression of Bcl-2 does not rescue the defect in T cell homeostasis observed in Jak3-deficient mice

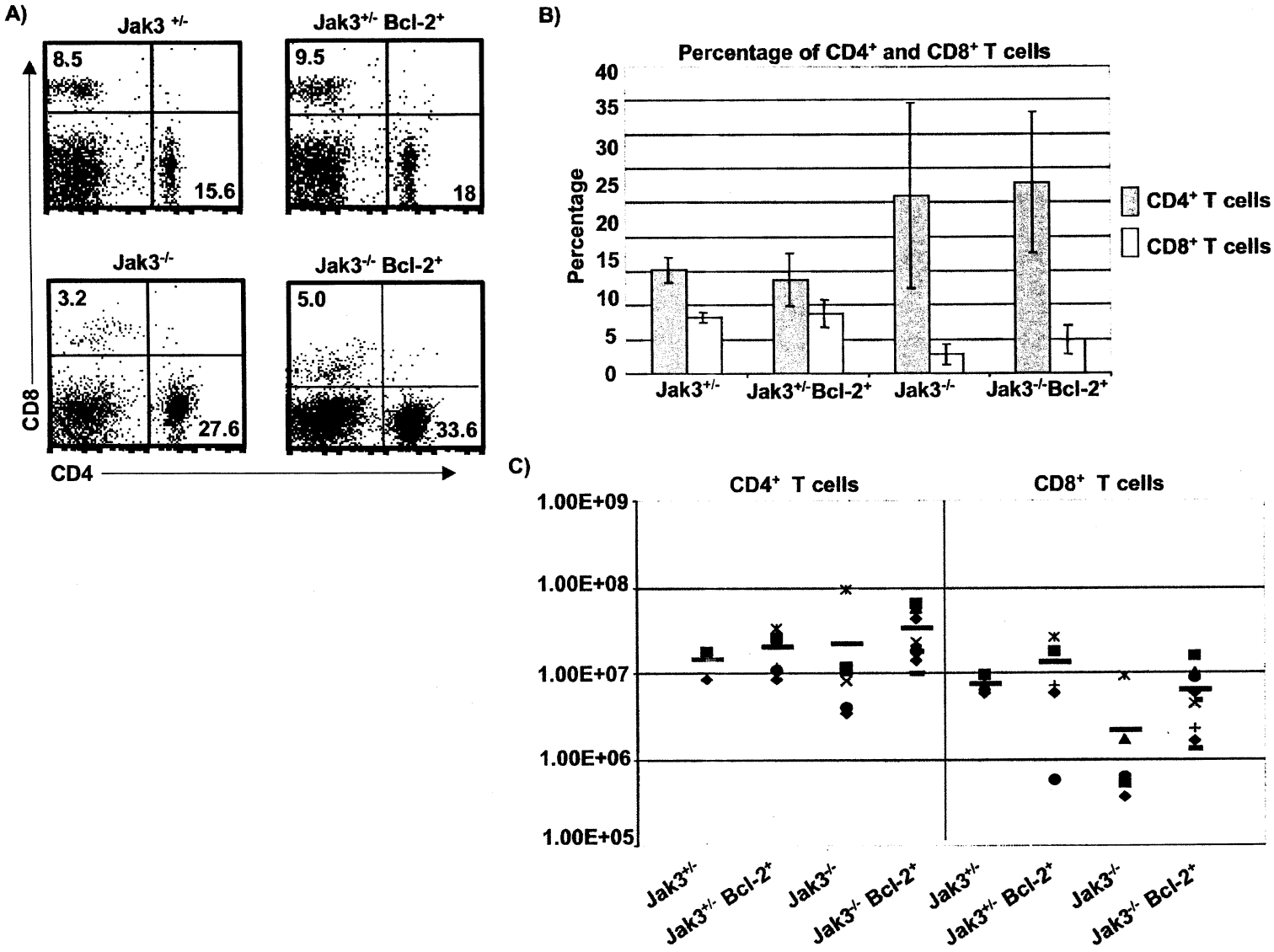
Next, I analyzed the total numbers and activation state of peripheral mature T cells. As reported before, the absence of Jak3/γ_c-mediated signals leads to an elevated CD4 to CD8 ratio and to the almost complete absence of $CD8^+$ mature T cells in the periphery of the mice. Survival of mature T cells is dependent on signals mediated by the IL-7R, which, among other possible effects, may lead to the upregulation of Bcl-2. When Maraskovsky et al crossed the $IL7^{-/-}$ mice to the Eμ-bcl-2 transgenic mice they reported that enforced expression of Bcl-2 resulted in a complete rescue of peripheral T cell numbers. I also investigated whether enforced expression of Bcl-2 could rescue the survival of naïve $CD4^+$ and $CD8^+$ T cells in Jak3-deficient mice.

In our system, expression of Bcl-2 led to an increase in the percentage and total numbers of $CD8^+$ T cells in the spleen of $Jak3^{-/-}bcl\text{-}2^+$ mice (Fig.13 A, B, C). However, these results varied considerably from mouse to mouse. The overall effect (accounting for all $Jak3^{-/-}bcl\text{-}2^+$ mice analyzed, n=10), was an increase in the percentage of peripheral $CD8^+$ T cells in $Jak3^{-/-}bcl\text{-}2^+$ of about 2-fold compared to $Jak3^{-/-}$ mice (Fig.13 B). In terms of total $CD8^+$ T cell numbers, the presence of Bcl-2 increased the mean from 2.18 x 10^6 in $Jak3^{-/-}$ mice to 6.38 x 10^6 in $Jak3^{-/-}bcl\text{-}2^+$ mice, compared to 7.5 x 10^6 and 1.34 x 10^7 in $Jak3^{+/-}$ and $Jak3^{+/-}bcl\text{-}2^+$ mice respectively (Fig.13 C). The percentage of mature $CD4^+$ T cells in $Jak3^{-/-}bcl\text{-}2^+$ mice remained relatively constant compared to $Jak3^{-/-}$ mice, and was elevated compared to $Jak3^{+/-}$ and $Jak3^{+/-}bcl\text{-}2^+$ mice. The total $CD4^+$ T cell numbers were slightly elevated in $Jak3^{-/-}bcl\text{-}2^+$ mice. The percentages and total numbers

of both $CD4^+$ and $CD8^+$ peripheral T cells remained relatively constant in $Jak3^{+/-}bcl\text{-}2^+$ mice compared to $Jak3^{+/-}$ control mice (Fig.13 A, B, C).

Fig.13 Slight increase in the percentage and total numbers of $CD8^+$ T cells in $Jak3^{-/-}$ $bcl\text{-}2^+$ mice

Total splenocytes from $Jak3^{+/-}$ (n= 3), $Jak3^{+/-}bcl\text{-}2^+$ (n= 7), $Jak3^{-/-}$ (n= 6), and $Jak3^{-/-}bcl\text{-}2^+$ (n= 10) were counted and stained for CD4 and CD8 expression. A) An example of the CD4 vs CD8 analysis for each genotypic model is depicted. B) The percentage of $CD4^+$ and $CD8^+$ T cells in the spleen of each mouse was calculated, and the averages of all the mice analyzed for each phenotypic group, as well as the standard deviations, are depicted. C) $CD4^+$ and $CD8^+$ total T cell numbers were calculated for each mouse. The red lines depict the average $CD4^+$ or $CD8^+$ T cell numbers for each genotypic group.


A)
Jak3 +/-
Jak3+/- Bcl-2+
Jak3-/-
Jak3-/- Bcl-2+
8.5
15.6
9.5
18
3.2
27.6
5.0
33.6
CD8
CD4
B)
Percentage of CD4+ and CD8+ T cells
Percentage
40
35
30
25
20
15
10
5
0
Jak3+/-
Jak3+/-Bcl-2+
Jak3-/-
Jak3-/-Bcl-2+
CD4+ T cells
CD8+ T cells
C)
CD4+ T cells
CD8+ T cells
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
Jak3+/-
Jak3+/- Bcl-2+
Jak3-/-
Jak3-/- Bcl-2+
Jak3+/-
Jak3+/- Bcl-2+
Jak3-/-
Jak3-/- Bcl-2+

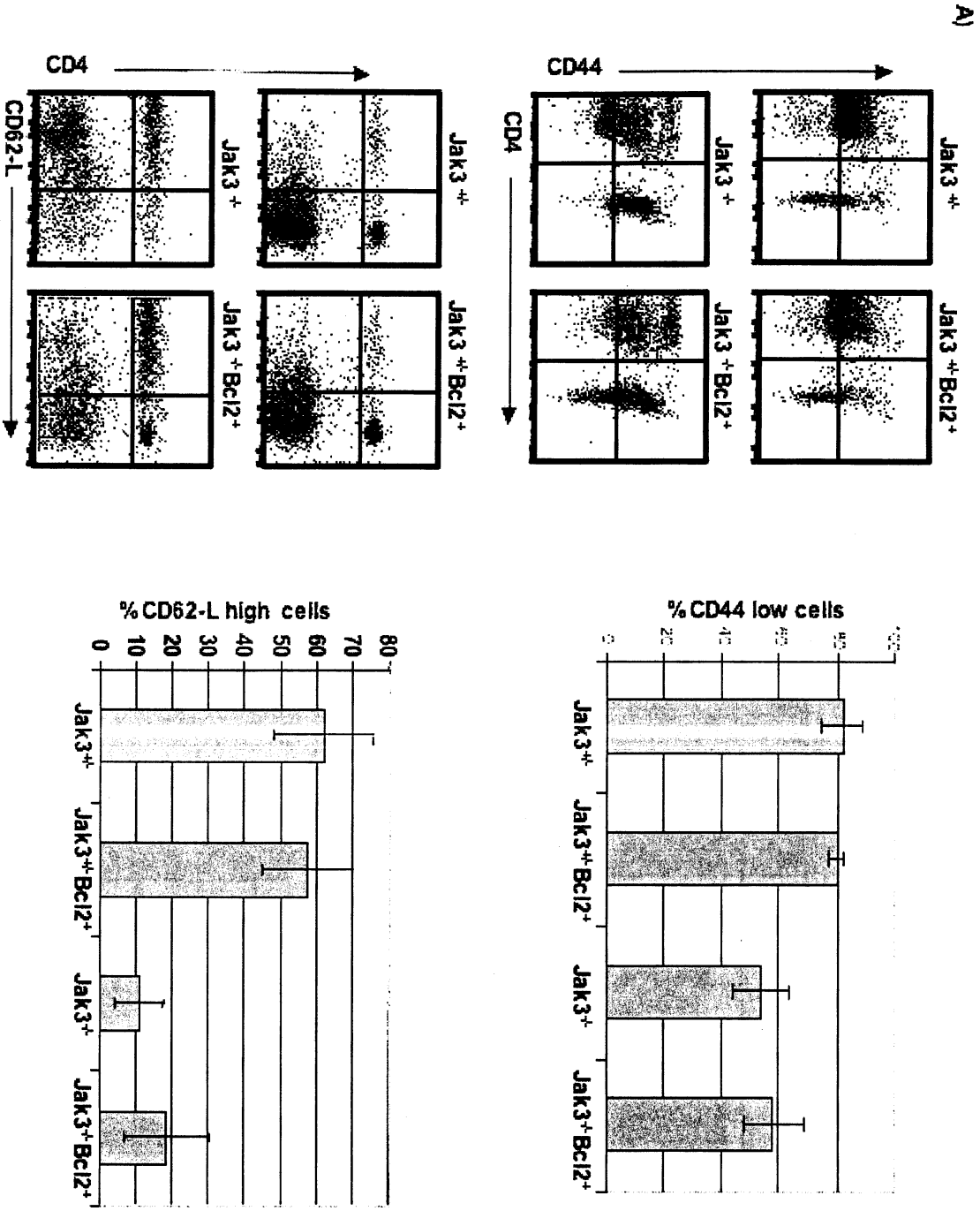
In addition to the dysregulation in total T cell numbers, the majority of T cells present in the periphery of Jak3/γ_c-deficient mice have an activated or memory-like phenotype. This may be due to the fact that memory $CD4^+$ T cells can survive better in the absence of γ_c cytokine signals, while both naïve $CD4^+$ and $CD8^+$ T cells, as well as memory $CD8^+$ T cells are more dependent on γ_c/Jak3-mediated signals to survive. Even if memory $CD4^+$ T cells are dying in the absence of IL-7-mediated signals, naïve $CD4^+$ T cells in $Jak3^{-/-}$ mice may be constantly receiving activation signals, due to the lack of regulatory $CD25^+$ T cells and defects in FasL upregulation, resulting in their proliferation, populating most of the periphery. Naïve $CD4^+$ and $CD8^+$ T cells from Jak3-deficient mice have low levels of Bcl-2 expression compared to $Jak3^{+/-}$ control mice. Therefore, I investigated whether the enforced expression of Bcl-2 resulted in the rescue of naïve $CD4^+$ and/or $CD8^+$ T cells in the periphery of Jak3-deficient mice.

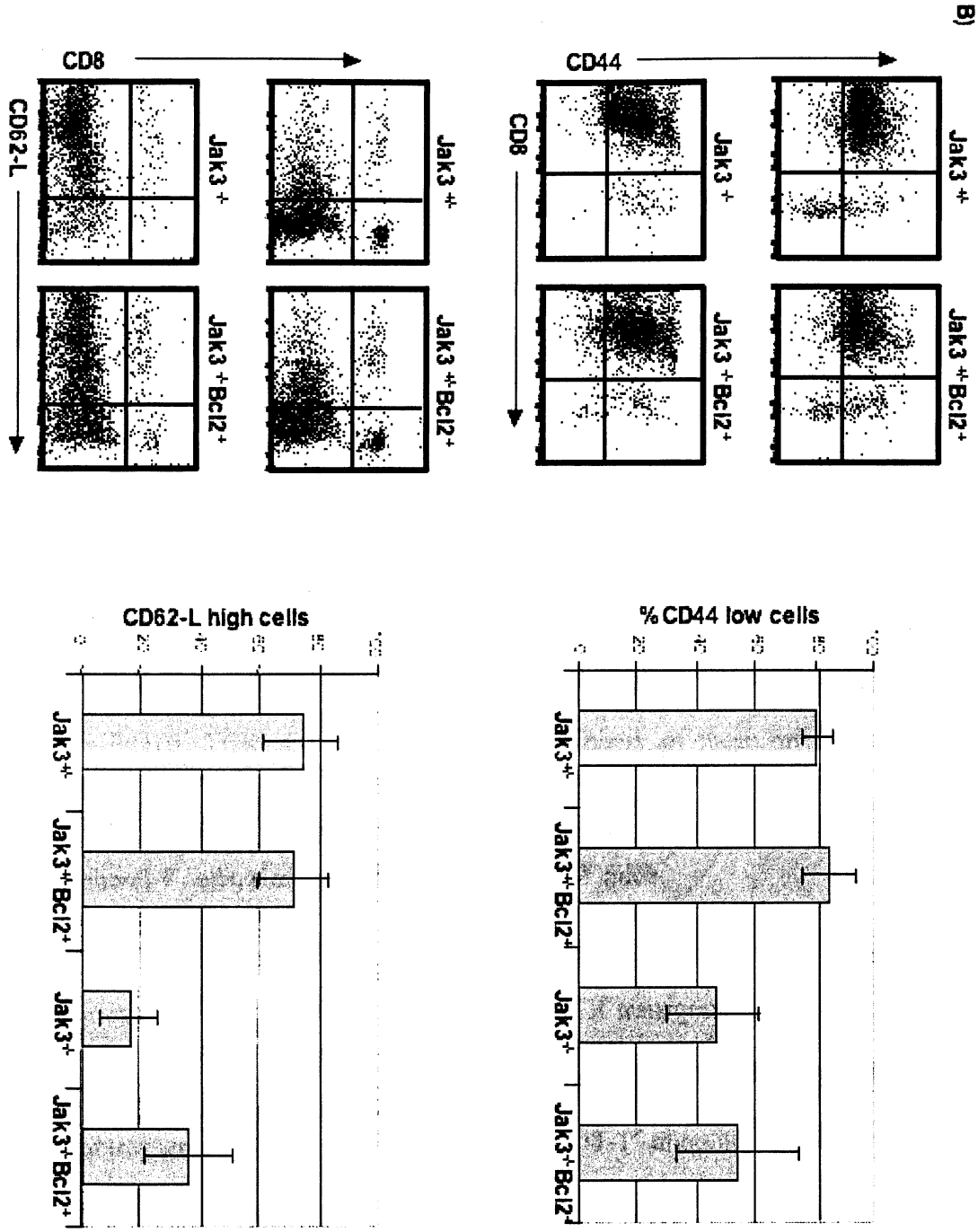
I stained splenocytes from $Jak3^{+/-}$, $Jak3^{+/-}$bcl-2^+, $Jak3^{-/-}$, and $Jak3^{-/-}$bcl-2^+ mice for the activation markers CD44 and CD62-L. Upon activation, T cells upregulate CD44 and downregulate CD62-L. As expected, the majority of mature $CD4^+$ and $CD8^+$ peripheral T cells from $Jak3^{+/-}$ mice presented a naïve phenotype characterized by the lack of CD44 and the expression of CD62-L. Only about 20%-30% of $CD4^+$ T cells presented an activated/memory-like phenotype (Fig.14 A). The enforced expression of Bcl-2 in $Jak3^{+/-}$ mice did not have a significant effect in the percentage of memory-like $CD4^+$ or $CD8^+$ T cells that populate the periphery of the mice (Fig.14 A, B). Contrary to these results, $Jak3^{-/-}$ mice had elevated percentages of memory-like T cells in the periphery. About 60% of the $CD4^+$ T cells were $CD44^{hi}$ (Fig.14 A). The CD44 staining usually appears as

a broad distribution rather than a clear division between high and low. Most of the $Jak3^{-/-}$ $CD4^+$ T cells were $CD44^{hi}$ or $CD44^{int}$ and very few were $CD44^{lo}$. About 90% of peripheral $CD4^+$ T cells in these mice expressed low levels of CD62-L. Similar results were observed with $CD8^+$ T cells (Fig.14 B). Although, the staining of these cells was not as clear, due to the very reduced numbers present in the spleens of $Jak3^{-/-}$ mice. When I analyzed the $Jak3^{-/-}bcl\text{-}2^+$ mice I was surprised to see that the percentages of activated T cells, both $CD4^+$ and $CD8^+$ T cells were very similar to those of the nontransgenic $Jak3^{-/-}$ mice (Fig.14 A, B). The vast majority of mature $CD4^+$ and $CD8^+$ T cells presented an activated phenotype. There was a slight increase in the percentage of $CD8^+$ naïve T cells, but overall more than 60% of the Bcl-2 transgenic $Jak3^{-/-}$ $CD8^+$ cells were $CD44^{hi}$ $CD\text{-}62L^{lo}$.

Fig.14 Enforced expression of Bcl-2 does not rescue the survival of naïve T cells in the periphery of Jak3$^{-/-}$ mice

Total splenocytes from Jak3$^{+/-}$, Jak3$^{+/-}$bcl-2^{+}, Jak3$^{-/-}$ and Jak3$^{-/-}$bcl-2^{+} were stained with α-CD4, α-CD8 and for activation markers with α-CD44 and α-CD62-L. An example of the FACS analysis for each phenotypic mouse model is depicted for both CD44 and CD62-L staining. A) The average percentages of gated naïve CD4^{+} T cells for all the mice analyzed in each genotypic group, as well as the standard deviations for each group of animals, are depicted in the graphs. The CD44 staining did not show a clear differentiation between CD44 positive and negative populations. The graph shows the percentage of CD4^{+} CD44^{hi} T cells and does not include the CD4^{+} CD44^{int} cells that are present in the FACS raw data. B) Similar analysis was done for the CD8^{+} T cells of each genotypic group. The staining in Jak3$^{-/-}$ mice is not as clear due to the low numbers of CD8^{+} T cells in the periphery of these mice


A)
CD44
CD4
Jak3 +/+
Jak3 -/-
Jak3 -/-Bcl2+
CD4
CD62-L
Jak3 +/+
Jak3 -/-
Jak3 -/-Bcl2+
% CD44 low cells
Jak3+/+
Jak3+/+Bcl2+
Jak3-/-
Jak3-/-Bcl2+
% CD62-L high cells
0
10
20
30
40
50
60
70
80
Jak3+/+
Jak3+/+Bcl2+
Jak3-/-
Jak3-/-Bcl2+


B)
CD44
CD8
Jak3 +
Jak3 +Bcl2+
CD8
CD62-L
Jak3 +
Jak3 +Bcl2+
% CD44 low cells
CD62-L high cells
Jak3+
Jak3+Bcl2+
Jak3+
Jak3+Bcl2+

B.3 Enforced expression of Bcl-2 can rescue a population of naïve $CD8^+$ mature T cells in Jak3-deficient OT-1 transgenic mice

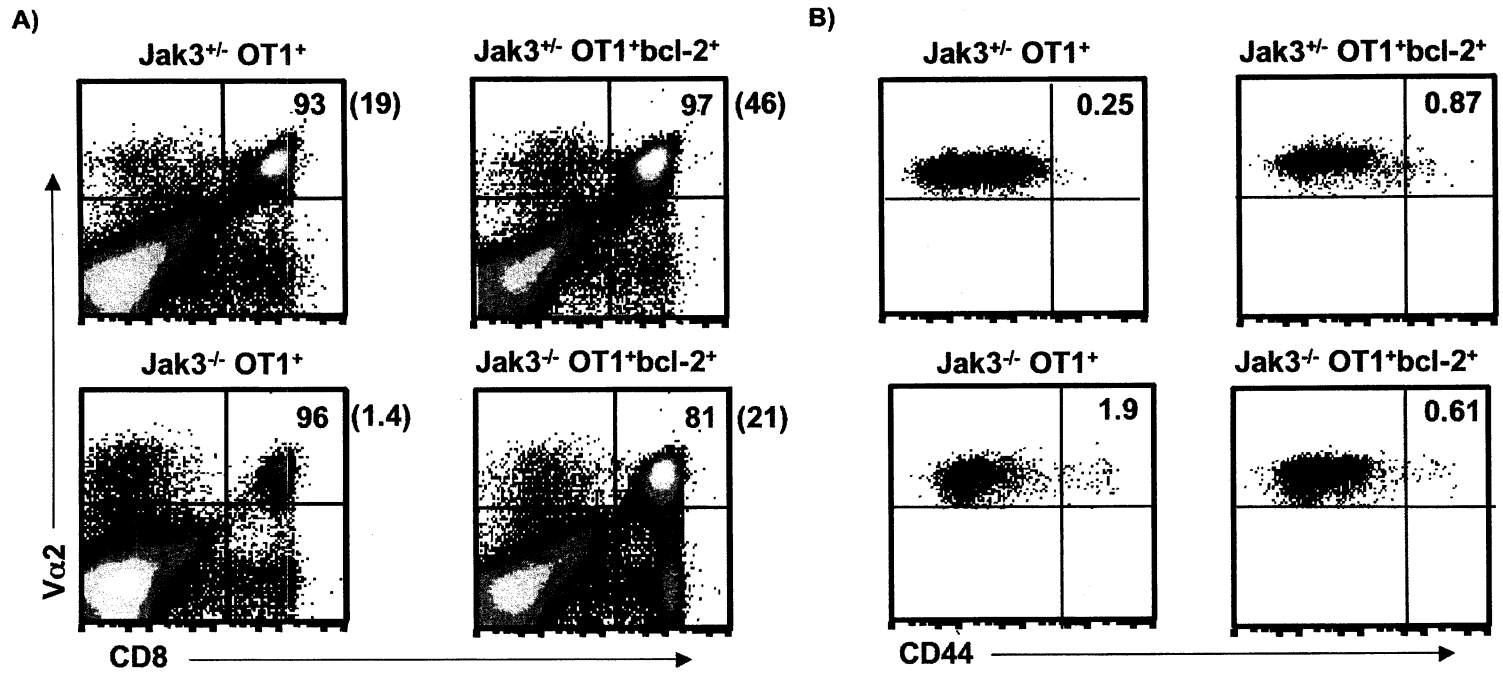
In previous studies Leonard's group had reported that enforced expression of Bcl-2 could rescue a substantial population of mature $CD4^+$ T cells in TCR transgenic γ_c-deficient mice. $Jak3^{-/-}$ and $\gamma_c^{-/-}$ mice have been crossed to several MHC-class I and MHC-class II restricted TCR transgenic mice, and in every case total the numbers of transgenic TCR^+ mature T cells are greatly reduced, compared to the numbers seen in transgenic TCR^+ WT mice. In their report, Leonard's group observed that the Bcl-2 expression could only rescue mature T cells when the TCR had a high affinity for self-MHC. The DO10 TCR has a high affinity for $H\text{-}2^{d/b}$ and when $\gamma_c^{-/-}$ $DO10^+bcl\text{-}2^+$ mice were crossed into this background, expression of Bcl-2 led to the production of a substantial population of mature $DO10^+$ TCR^+ $CD4^+$ T cells in the periphery of these mice. In the $H\text{-}2^{d/d}$ background, for which the DO10 TCR has a low affinity, $\gamma_c^{-/-}bcl\text{-}2^+DO10^+$ mice had very few peripheral T cells, similar to the $\gamma_c^{-/-}DO10^+bcl\text{-}2^-$ mice.

I decided to analyze the effect of enforced expression of Bcl-2 in Jak3-deficient mice that expressed an MHC-class I restricted TCR transgenic line. I chose the OT-1 TCR, which recognizes the SIINFEKL peptide of the ovalbumin protein bound to K^b. When nontransgenic $Jak3^{-/-}$ mice were crossed to $OT\text{-}1^+$ mice, the numbers of peripheral $CD8^+$ TCR^+ T cells were low and very reduced compared to $Jak3^{+/-}$ $OT\text{-}1^+$ mice (Fig.15 A). However, crossing the $Jak3^{-/-}$ $OT\text{-}1^+$ mice to the Eμ-bcl-2 transgenic mice I observed a large $OT\text{-}1^+$ $CD8^+$ mature T cell population (Fig.15 A). The percentage and total numbers of $CD8^+$ T cells in $Jak3^{-/-}OT\text{-}1^+bcl\text{-}2^+$ mice were reduced compared to both

Jak3$^{+/-}$OT-1^{+}bcl-2^{-} or bcl-2^{+}, but they were significantly higher than those observed in Jak3$^{-/-}$OT-1^{+}bcl-2^{-} mice (data not shown). Furthermore, the expression of CD44 (Fig.15 B) and CD62L (data not shown) revealed that a majority of the CD8^{+} TCR^{+} T cells in the spleen of Jak3$^{-/-}$OT-1^{+}bcl-2^{+} mice had a naïve phenotype. This discovery was especially exciting as it provided me with a large population of mature naïve CD8^{+} T cells that were deficient in Jak3, and whose specificity was known. This mouse model presented me with the opportunity to investigate the ability of CD8^{+} T cells to mount an immune response in the absence of γ_c cytokine signals.

Fig.15 Enforced expression of Bcl-2 rescues a naïve population TCR^+ $CD8^+$ T cells

$Jak3^{-/-}$and $Jak3^{-/-}$bcl-2^+ mice were crossed to OT-1 TCR transgenic mice. The resulting $Jak3^{+/-}$bcl-2^+OT-1^+ mice were intercrossed to obtain $Jak3^{-/-}$bcl-2^+OT-1^+ and littermate control $Jak3^{+/-}$bcl-2^+OT-1^+ mice, as well as $Jak3^{-/-}$OT-1^+ mice and littermate control $Jak3^{+/-}$OT-1^+ control mice. Mice of the four different phenotypes were sacrificed and their spleens stained for CD8, Vα-2 (expressed by the OT-1 TCR) and the activation marker CD44. A) The percentages of OT-1^+ ($V\alpha2^+$) cells within the $CD8^+$ population are depicted within the FACS plot, the percentages of TCR^+ $CD8^+$ cells in the spleen of each mouse phenotype are depicted in parenthesis outside the FACS plot. B) The numbers depict the expression of $CD44^{hi}$ in Vα-2^+ $CD8^+$ gated T cells.


A)
Jak3+/- OT1+
93
(19)
Jak3+/- OT1+bcl-2+
97
(46)
Jak3-/- OT1+
96
(1.4)
Jak3-/- OT1+bcl-2+
81
(21)
Vα2
CD8
B)
Jak3+/- OT1+
0.25
Jak3+/- OT1+bcl-2+
0.87
Jak3-/- OT1+
1.9
Jak3-/- OT1+bcl-2+
0.61
CD44

C. DISCUSSION

Signals downstream of the γ_c chain and Jak3 are extremely important for the development of lymphocytes. In mice, the absence of γ_c cytokines leads to the arrest of B-cell development at the pro-B cell stage. Likewise, γδ T cells and NK cells do not mature in the thymus of mice lacking γ_c- or Jak3-mediated signals. Mouse models in which single γ_c cytokines have been eliminated have provided me with some insight on the specific cytokine/s that play a role during each stage of development, in each lymphocyte subset. IL-7 appears to play an essential role in T cell development. The developmental defects observed in the thymi of IL-7R$^{-/-}$ and $\gamma_c^{-/-}$ or Jak3$^{-/-}$ are very similar. They all have small thymi with extremely reduced cellularity. While the development of γδ T cells is completely blocked in the thymus, αβ thymocytes, though reduced in cellularity, appear to develop normally as demonstrated by the presence of normal proportions of thymocyte populations at the different developmental stages.

In order to better understand the defect observed in thymic development in the absence of IL-7-mediated signals, IL-7R-deficient mice were crossed to Bcl-2 transgenic mice that express Bcl-2 under the Eμ promoter. Using this model, Weissman's group observed that the number of thymocytes were increased 10-fold compared to the IL-7Rα$^{-/-}$ mice, making the cellularity about 20% that of a normal thymus [248]. Maraskovsky et al saw an even more profound rescue in thymocyte numbers, to practically normal levels, using the same experimental system [249]. The reasons behind the discrepancy, in terms of the levels of rescue obtained by each group, have not been elucidated. Both groups, however, claimed that enforced expression of Bcl-2 was enough to rescue the

developmental defect observed in mice deficient for IL-7-mediated signals. Crossing γ_c-deficient mice to different Bcl-2 transgenic mouse models has also generated conflicting results. Weissman's group saw the most striking rescue in thymocyte development when $\gamma_c^{-/-}$ mice were crossed to transgenic mice that expressed Bcl-2 under the H-2K promoter [18]. The total numbers of thymocytes in these mice were 50% those of the WT control mice. Contrastingly, Rodewald et al saw a very small rescue when they crossed $\gamma_c^{-/-}$ mice to transgenic mice that express Bcl-2 under the Eμ promoter, and likewise, Leonard's group saw a very limited rescue in thymocyte development when they crossed $\gamma_c^{-/-}$ mice to transgenic mice that express Bcl-2 under the Lck promoter [17, 250]. When I crossed $Jak3^{-/-}$ mice, which are phenotypically identical to γ_c-deficient mice, to transgenic mice that express Bcl-2 under the Eμ promoter, I also saw a very limited rescue in thymocyte development. Despite the argument that the Eμ promoter may not be expressed in the earliest thymocyte precursors, Rodewald et al showed that enforced expression of Bcl-2 under this promoter could rescue WT thymocytes from dying *in vitro* from cytokine withdrawal apoptosis [18, 250]. Additionally, O'Reilly et al had previously shown that, under the Eμ promoter, Bcl-2 was expressed in all TN populations (TN1-TN4) and its expression promoted the survival of pro-T cells incubated in the absence of added cytokines [253]. Further, using the Lck promoter to drive the expression of Bcl-2, Leonard's group also failed to see a significant rescue in thymocyte development [17].

When I analyzed the TN stages of development I detected that the block observed in γ_c-deficient and Jak3-deficient mice, between the TN2 and TN3 stages, was not bypassed by the enforced expression of Bcl-2. These results suggest that the

developmental block observed in Jak3$^{-/-}$ mice could not be rescue by Bcl-2 alone. These results are consistent with Rodewald's results upon analysis of the same developmental populations. Weissman's group failed to analyze the TN stages of development in either the Eμ, or the H-2K promoter systems. Therefore, it is possible that the changes observed by this group upon enforced expression of Bcl-2 simply resulted in the survival of more thymocytes, instead of an actual rescue in thymocyte development.

These previous studies have also reported controversial results about the ability of Bcl-2 to rescue peripheral T cell numbers in the absence of γ_c-mediated signals. Weissman's group reported a 3-fold increase (to 20% of WT mice), using the Eμ promoter, and a 10-fold increase (to 50%), using the H-2K promoter in the blood of Bcl-2 transgenic $\gamma_c^{-/-}$ mice, somewhat restoring the numbers of peripheral T cells to normal levels. Furthermore they reported that the CD4 to CD8 ratio, which appears elevated in $\gamma_c^{-/-}$ mice, was restored to normal. The authors did not report any results on the activation status of mature T cells found in the periphery of these mice. When I looked at the periphery I observed that the total numbers were variable from mouse to mouse, both in nontransgenic Jak3$^{-/-}$ and Jak3$^{-/-}$bcl-2^{+} mice. The percentage and numbers of CD4^{+} T cells were comparable between Jak3$^{-/-}$ and Jak3$^{-/-}$bcl-2 mice, and most of them presented an activated or memory-like phenotype. The percentage and numbers of CD8^{+} T cells was increased about 2-fold by the enforced expression of Bcl-2 compared to Jak3$^{-/-}$ mice. However, neither the percentage nor the total number of mature CD8^{+} T cells ever increased to WT levels. Most CD8^{+} mature T cells in both animal models presented an activated/memory phenotype. As observed in Jak3$^{-/-}$ and $\gamma_c^{-/-}$ mice, the percentage of

$CD4^+$ T cell was higher in both $Jak3^{-/-}$ and $Jak3^{-/-}bcl\text{-}2^+$ mice compared to WT control mice.

Due to the small size of the thymus in the $Jak3^{-/-}$ and $Jak3^{-/-}bcl\text{-}2^+$ mice, the input of immature T cells into the periphery of these mice is very limited. Additionally, naïve T cells require IL-7 and memory $CD8^+$ T cells require IL-15 in order to survive. Bcl-2 may be one of the factors that contribute to the survival of mature cells, but other survival or cell cycle factors may also play an important role. For example, Bcl-xL was found to mediate the survival of developing $CD8^+$ T cells in the thymus [190]. Alternatively, it is possible that, due to the small number of mature cells entering the periphery, a fraction of T cells may be undergoing homeostatic proliferation, leading to the accumulation of memory-like T cells in these mice, preventing any accumulation of naïve cells that would otherwise survive under the enforced expression of Bcl-2. Memory-like $CD4^+$ T cells do not require Jak3/γ_c-mediated signals to survive for long periods of time, or to homeostatically proliferate. Therefore, if a small fraction of mature $CD4^+$ T cells receive an activation signal, not only will they be able to survive in the absence of γ_c cytokines, but they should also be able to homeostatically proliferate, populating the majority of the periphery. Furthermore, the lack of IL-2R signaling in activated/memory $CD4^+$ T cells in these mice may prevent them from undergoing FasL-mediated AICD. In contrast, $CD8^+$ T cells require IL-15- and to a lesser extent IL-7-mediated signals to survive and homeostatically proliferate. I therefore reasoned that, in the absence of mature T cells that may undergo this activation-induced or homeostatic proliferation, naïve T cells may be able to accumulate in the periphery of $Jak3^{-/-}bcl\text{-}2^+$ mice.

In order to do this, I decided to cross these mice to a TCR transgenic mouse. I chose the OT-1 transgenic system. The OT-1 mice had already been crossed to straight $Jak3^{-/-}$ mice in our lab. Analysis of $Jak3^{-/-}$ $OT\text{-}1^{+}$ mice revealed that the thymi were greatly reduced in cellularity, and that the numbers of peripheral T cells were extremely low. Most of the peripheral T cells that expressed the OT-1 transgene were naïve, whereas T cells that expressed endogenous α chains appear to have a memory-like phenotype. However, the T cell numbers were highly reduced compared to $Jak3^{-/-}$ mice [204]. When I crossed the OT-1 mice to the Eμ-bcl-2 transgenic mice I observed that the thymi were still greatly reduced in cellularity but that the percentage and numbers of $OT\text{-}1^{+}CD8^{+}$ T cells in the periphery of $Jak3^{-/-}OT\text{-}1^{+}bcl\text{-}2^{+}$ mice were considerably higher than those observed in the $Jak3^{-/-}OT\text{-}1^{+}$ mice. The percentage and numbers were still lower than the $OT\text{-}1^{+}Jak3^{+/-}$ control mice, but there was a significant population of mature T cells that also appeared to have a naïve phenotype. I reasoned that, in the absence of endogenous $CD4^{+}$ T cells that could proliferate and populate the periphery, mature $CD8^{+}$ T cells that expressed Bcl-2 were able to accumulate in these mice. Additionally, the OT-1 TCR is known to have high affinity for self-MHC-self-peptide complexes that may have permitted these cells to survive in the presence of an anti-apoptotic factor. Of particular importance was the fact that this system provided me with a population of $CD8^{+}$ naïve T cells of known specificity to investigate the ability of Jak3-deficient $CD8^{+}$ T cells to mount immune responses. Because of the lower numbers of $CD8^{+}$ T cells present in the periphery of Jak3-deficient and Jak3-deficient TCR transgenic mice, experiments of this type were not previously possible.

In summary, I have demonstrated that enforced expression of Bcl-2 using the Eμ promoter cannot rescue the block observed in Jak3-deficient thymocyte maturation between the TN2 and TN3 stages of development. Bcl-2 expression does not rescue thymocyte numbers to normal levels. Additionally Bcl-2 does not rescue the T cell homeostasis defect observed in the periphery of mice deficient in Jak3-mediated signals. $Jak3^{-/-}bcl\text{-}2^{+}$, like $Jak3^{-/-}$ mice, have an elevated CD4 to CD8 ratio, the percentage and numbers of CD8 T cells are reduced, and most of the mature T cells ($CD4^{+}$ and $CD8^{+}$) appear to have an activated/memory phenotype. This may be due to the selective survival of activated or homeostatically expanded $CD4^{+}$ $CD44^{+}$ T cells that are independent of Jak3/γ_c-mediated signals. However, in the absence of $CD4^{+}$ T cells that could receive a TCR-mediated signal to expand, Bcl-2 can rescue a significant $CD8^{+}$ TCR transgenic mature T cell population.

CHAPTER V

ANTIVIRAL IMMUNE RESPONSES ARE INITIATED BUT NOT SUSTAINED BY T CELLS LACKING JAK 3-MEDIATED CYTOKINE SIGNALS

A. INTRODUCTION

A.1 γ_c cytokines play an important role during $CD8^+$ T cell activation

T cell activation is a fascinating and tightly controlled mechanism that protects us against innumerable pathogens in an incredibly efficient manner. The diverse TCR repertoire expressed by mature $CD4^+$ and $CD8^+$ T cells in mammals, allows them to recognize most any infecting pathogen throughout the lifetime of the organism. Signals initiated upon the binding of a TCR with its specific self-MHC-peptide complex, in addition to signals mediated by costimulatory molecules expressed by T cells, lead to T cell activation and differentiation into effector cells, and the initiation of an adaptive immune response [91, 92, 94-96]. Only a small population of T cells expresses the TCRs that can recognize a given immunogen, however, due to the clonal expansion that follows T cell activation, the restricted number of specific T cell clones is enough to create an effective immune response, which results in the clearance of the infection [52, 97, 102]. T cell activation and expansion is followed by the apoptosis of most of the activated T cells and the formation of a small memory T cell population, restoring the homeostatic state characteristic of an infection-free environment [52, 96].

In addition to the TCR-mediated and costimulatory signals, other pathways, which include the production of cytokines and signals through their receptors, also play important roles during T cell activation [108-110]. The family of cytokines that signals through the cytokine receptor common gamma chain, i.e., IL-2, -4, -7, -9, -15, and –21, are of particular importance [209-211]. However precise and controlled, the system is sometimes faulty and under certain circumstance may lead to inefficient responses that

lead to the incomplete clearance of an immune challenge, or efficient responses against self-molecules that lead to auto-immunity. Understanding the many facets that contribute to the creation of T cell immunity is essential for our knowledge of basic immunology as well as the development of vaccines and immunoregulatory agents.

Despite their importance, the function of γ_c/Jak3-dependent cytokine signals in T cell activation, proliferation and differentiation has been difficult to discern. Functional cytokine redundancy, together with their essential role in T cell survival, has made it extremely difficult to assess their function during T cell activation and differentiation. Additionally, mice lacking γ_c or Jak3 have virtually no peripheral $CD8^+$ T cells. This lack of peripheral $CD8^+$ T cells is also observed when γ_c or Jak3-deficient mice are crossed to appropriate TCR transgenic lines [203, 204]. Therefore, a common approach has been to study mice that are deficient in individual cytokine or cytokine receptors of the γ_c family. The results remain controversial. Some of the studies have reported that deficiencies in one or two of these cytokines have relatively minor effects on T cell activation and function. For example, it is widely accepted that IL-2 is a major T cell growth factor, and that it plays a critical role in the differentiation of naïve T cells into effector cells. Yet, when IL-2-deficient mice were infected with LCMV or vaccinia, they were able to generate protective immunity to both viruses, producing both CTL and anti-viral antibody responses [207]. IL-4-mediated signals were shown to compensate for the lack of IL-2, suggesting that other cytokines might play a compensating role in IL-2-deficient mice, exemplifying the difficulties in determining the precise function of cytokine signals for productive immune responses. Additional studies using mice

deficient in IL-2 and IL-15 receptor signals (IL-2Rβ$^{-/-}$) also suggested that the two cytokines are dispensable for T cell-mediated immune responses but that the magnitude and effector response is sometimes impaired [208]. The amount of IFN-γ secreted by cells from IL-2Rβ$^{-/-}$ mice in response to a vaccinia infection was markedly reduced, in addition to their inability to elicit a CTL response *in vitro*, due to reduced induction of perforin and granzyme B [208].

In contrast, Cousens et al reported that the absence of IL-2 led to a dramatic inhibition of CD8$^+$ T cell expansion following LCMV infection [210]. The percentages of CD8$^+$ T cells did not increase unless IL-2 was added during the infection. Further, the production of IFN-γ was significantly reduced at the peak of the immune response in IL-2$^{-/-}$ mice [210]. Recent studies, using the OT-1 TCR transgenic model, have suggested that in the absence of IL-2, CD8$^+$ T cells can initiate a specific immune response, but they cannot undergo the subsequent proliferative expansion observed in wild type mice [211]. Finally, infection of IL-15 knock-out mice with VSV resulted in a reduction in the number of tetramer positive CD8$^+$ T cells compared to the wild type controls. The decrease was only obvious after day 6 of infection, suggesting that IL-15 was important in determining the amplitude of the VSV-specific primary response [212]. These mice were also deficient in generating a substantial portion of antiviral CD8$^+$ memory T cells [212].

Overall, the requirements for γ_c cytokines during CD8$^+$ T cell responses remain controversial and our knowledge has derived solely from experiments focused on individual cytokines or cytokine receptors. In this chapter I describe studies I have

conducted in order to better understand the role that cytokines of the γ_c chain play during $CD8^+$ T cell-mediated immune responses.

A.2 Experimental systems of T cell activation

In order to gain an understanding of T cell mediated immune responses, different experimental systems have been developed, allowing me to follow infections *in vivo* and to help me elucidate the many factors that play important roles during T cell responses. One of these systems, arguably one that has given us the most knowledge of $CD8^+$ T cell immunity, is the lymphocytic choriomeningitis virus (LCMV) infection of mice [254]. Infection of C57BL/6 mice with the Armstrong strain of LCMV leads to the production of type I interferons during the early stages of virus replication, which initiates the activation of NK cells. After the first few days of infection specific $CD4^+$ and $CD8^+$ T cells begin to expand and peak in numbers about 8 to 9 days post infection [255, 256]. $CD8^+$ T cells differentiate into effector CTLs capable of controlling the viral titers that begin to decline even before the peak of the CD4-mediated T helper response [257]. CTLs contain granzymes and perforin, which are essential for the successful development of an LCMV acute response [258, 259]. These cells also secrete antiviral cytokines, IFN-γ and TNF-α, and express FasL, which allows the cells to lyse Fas-expressing infected targets [119, 254]. A very important advantage of the LCMV system is that immunodominant and subdominant LCMV epitopes have been defined for both $CD4^+$ and $CD8^+$ specific T cells [218, 219, 260-262]. Following virus clearance activated T cells become sensitive to apoptosis and their numbers decline, leaving the host in a

steady-state of T cell homeostasis with a stable memory population that will provide lifelong immunity to the virus [263, 264].

Another essential system for the understanding of T cell mediated immunity is the adoptive transfer of TCR transgenic T cells into host mice [52, 97]. As mentioned before, a very small number of T cells recognize a given pathogen under normal circumstances. The numbers are so low that they are impossible to detect in the periphery of a healthy naïve mouse. On the other hand, the numbers of specific T cells in the periphery of a TCR transgenic mouse are too high to be physiologically relevant. Therefore, it has been very difficult to follow a specific population of T cells from their naïve state under normal circumstances, to their activated state upon antigen challenge, to their apoptosis and the formation of a memory population. The solution to this problem is the adoptive transfer of TCR transgenic T cells into congenic normal recipients. This system takes advantage of the existence of T cells from TCR transgenic mice that are specific for a known MHC-peptide complex. By taking a small number of these T cells from the secondary lymphoid organs of the transgenic mice, and transferring them i.v. into a congenic non-TCR transgenic host, the cells can be followed before, during and after the injection of the specific antigen into the naïve host [52]. Using this system, the population of TCR transgenic T cells in the naïve host is large enough to be detected with anti-clonotypic Abs, but small enough to behave in a physiological manner. The host mice can then be injected with the antigen that is specifically recognized by the transgenic TCR and the immune response can be followed in a time course.

I have taken advantage of both the LCMV and the adoptive transfer systems to study the importance of γ_c cytokines during the initiation and development of a $CD8^+$ T cell mediated immune response. I have infected Jak3-deficient mice that lack all γ_c cytokine-mediated signals with LCMV and studied their ability to mount and appropriate immune response, and clear the virus. Jak3-deficient mice have very reduced numbers of mature $CD8^+$ T cells and their TCR repertoire may be skewed, as I saw was the case for $CD4^+$ T cells [265], thus it is possible that these mice lack LCMV specific T cells. Therefore, if I were to observe a defect in the ability of these mice to mount an appropriate immune response, it could be due to the absence of specific T cells and not to the inability of those T cells to respond to an antigenic challenge. Consequently, I have designed experiments in which the same number of TCR transgenic $CD8^+$ T cells, from mice that express or lack Jak3, are stimulated by their specific antigen under similar conditions, both *in vitro* and *in vivo*. These experiments directly address the ability of Jak3-deficient $CD8^+$ T cells to mount an immune response. As described in the previous chapter, when Jak3 deficient mice are crossed to OT-1 transgenic mice, the numbers of peripheral $CD8^+$ T cells are extremely small, definitely not large enough to conduct appropriate studies. However, when the $Jak3^{-/-}$ $OT\text{-}1^+$ mice are crossed to Bcl-2 transgenic mice, a distinct population of naïve $CD8^+$ T cells that express the transgenic TCR is rescued in the spleen of these mice. This model has allowed me to conduct adoptive transfer experiments, as well as *in vitro* activation experiments. Remarkably, the data from the two independent systems are consistent, and demonstrate that γ_c/Jak3-

dependent cytokine signals are required to maintain, but not to initiate, anti-viral immune responses.

B. RESULTS

B.1 Jak3$^{-/-}$ mice can mount a weak and non-protective CD8^{+} T cell response to LCMV infection

To evaluate Jak3 signaling during a CD8^{+} T cell response, I examined the ability of Jak3$^{-/-}$ mice to respond *in vivo* against a viral challenge. As a first approach, I chose to infect Jak3$^{-/-}$ and control (Jak3$^{+/-}$) mice with LCMV. In wild type mice, LCMV infection elicits a robust CD8^{+} T cell response that is first evident at day 5 after infection, peaks at day 8-9, and then declines [255, 256]. There are several advantages to utilizing this well-characterized system. First, viral clearance of the Armstrong strain of LCMV is dependent on CD8^{+} T cell function alone, and does not require help from CD4^{+} T cells; thus, anti-viral CD8^{+} T responses can be assessed without confounding effects caused by potentially impaired CD4^{+} T cell responses in Jak3$^{-/-}$ mice [266, 267]. In addition, the immunodominant LCMV-specific CD8^{+} T cell epitopes in C57BL/6 mice have been identified, thus providing a means to examine both the frequency and the function of responding cells [218, 219, 260-262]. Finally, at their peak, virus-specific CD8^{+} T cells represent >80% of the total CD8^{+} T cell pool, resulting from a >1000-fold expansion in virus-specific cells [268]. This dramatic expansion observed in wild type mice increased the likelihood that I might observe a response, even a severely diminished one, in Jak3$^{-/-}$ mice.

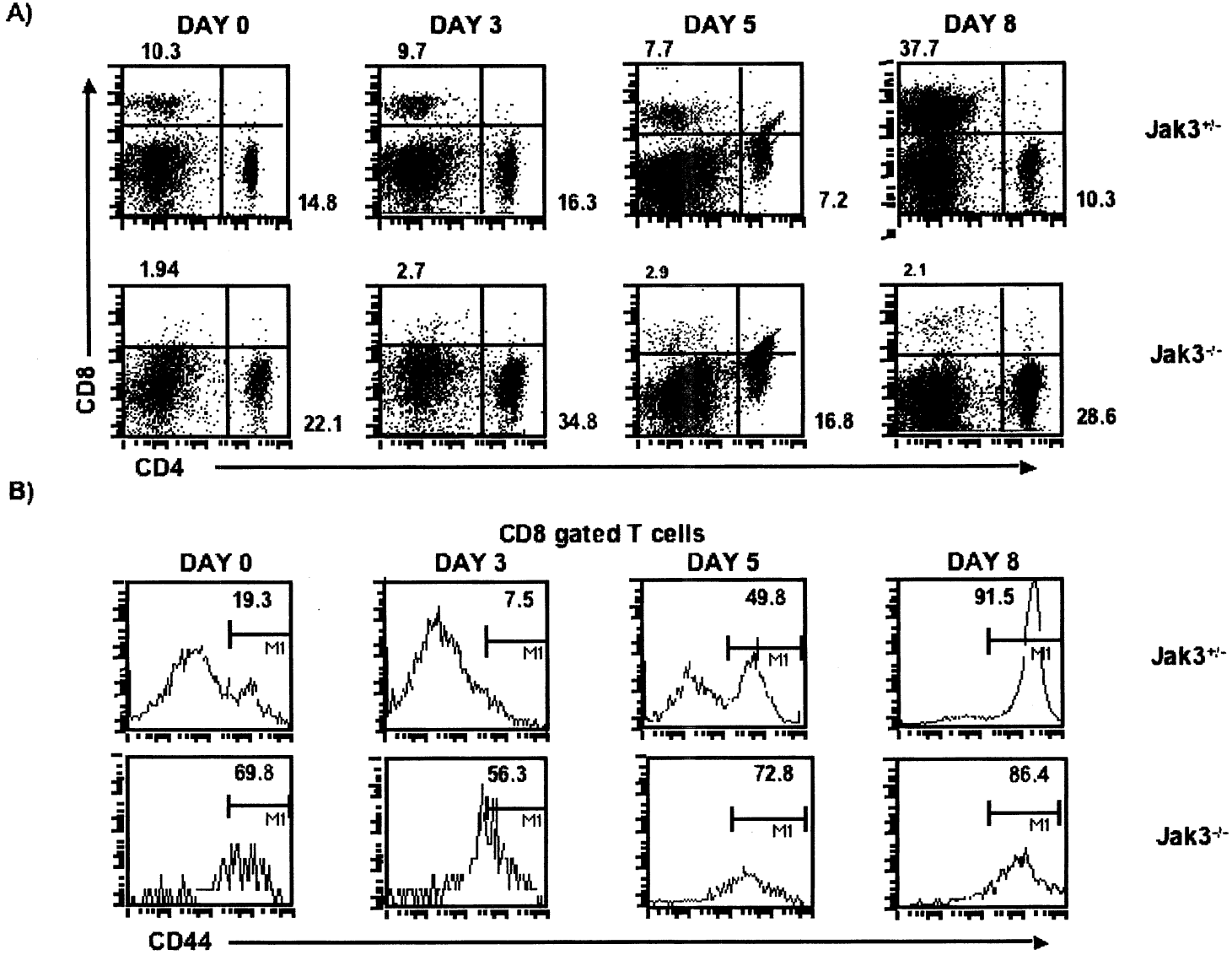
Several aspects of the response to LCMV were examined, including the ability of the $CD8^+$ and $CD4^+$ T cells to become activated, to proliferate, and to produce IFN-γ in response to re-stimulation ex vivo. As shown in Figure 16 A, splenocytes from uninfected $Jak3^{-/-}$ mice (day 0) have an increased proportion of $CD4^+$ T cells compared to controls, but relatively few $CD8^+$ T cells. Furthermore, most of the $CD8^+$ T cells in the $Jak3^{-/-}$ mice have an activated/memory cell phenotype, as indicated by their high surface expression of CD44 (Fig.16 B). Mice were then infected with LCMV (Strain Armstrong), and responses were examined on days 3, 5, and 8 following infection. For the $Jak3^{+/-}$ mice, the sequence of events followed the expected pattern. At day 3, there was a noticeable reduction in the percentage of $CD8^+$ T cells in the spleen that are $CD44^{hi}$, an attrition that has been attributed to the effects of type I interferons, as previously described [269]. By day 5 following infection, the percentage of $CD8^+$ T cells in the $Jak3^{+/-}$ were slightly reduced compared to day 0, but the total numbers of $CD8^+$ T cells were increased by ~3-fold (data not shown). At the peak of the immune response (day 8), the percentage and total number of $CD8^+$ T cells in $Jak3^{+/-}$ mice was extremely high (~15-fold increase in absolute cell numbers over day 0) and nearly all of the cells had an activated phenotype (Fig.16 A, B).

In contrast, only a very modest or no response was observed in $Jak3^{-/-}$ mice following LCMV infection. Only 40% of the animals (n=24) were able to mount a $CD8^+$ T cell response as measured by $CD8^+$ T cell expansion and IFN-γ production. Figure 16 shows a representative example of the mice that responded to the virus. The percentage of $CD8^+$ T cells in the spleen did not increase significantly, but at day 5 and 8 post-

infection a clear population of $CD8^+$ cells was visible, in contrast to the profiles seen in non-infected $Jak3^{-/-}$ mice. This population was not visible in the mice that did not present a $CD8^+$ effector T cell population (data not shown). Furthermore, by day 5 post-infection, there was a detectable increase in the absolute numbers of $Jak3^{-/-}$ $CD8^+$ T cells (~2-fold over day 0). No further increase in $CD8^+$ T cell numbers occurred between day 5 and day 8 post-infection, even in the responding $Jak3^{-/-}$ mice, a time at which the most dramatic expansion in wild type anti-viral $CD8^+$ T cells is observed. Finally, there was little change in the percent of $CD44^{hi}$ $CD8^+$ T cells in $Jak3^{-/-}$ mice over the course of the viral infection. Together, these data indicate that γ_c/Jak3-dependent cytokine signals are not absolutely required for $CD8^+$ T cell proliferation in response to infection, but that the magnitude of the proliferative response is greatly diminished in their absence.

Fig.16 Limited Jak3$^{-/-}$ CD8^{+} T cell proliferation in response to LCMV infection

Jak3$^{-/-}$ and Jak3$^{+/-}$ control mice were injected i.p. with 4.0 x 10^4 PFUs of LCMV Armstrong. Spleens from infected, as well as uninfected control mice, were isolated at different time points following LCMV infection. A) Splenocytes were counted and stained with CD4 and CD8, as well as B) the activation markers CD44 and CD62-L (data not shown) mAbs. The numbers represent A) percent of CD4 and CD8 T cells in spleen or B) percent of activated (CD44^{hi}) CD8^{+} T cells at each given timepoint. The FACS plots are representative of n=6 mice for day 3 and 5, and n=24 for day 0 and 8.

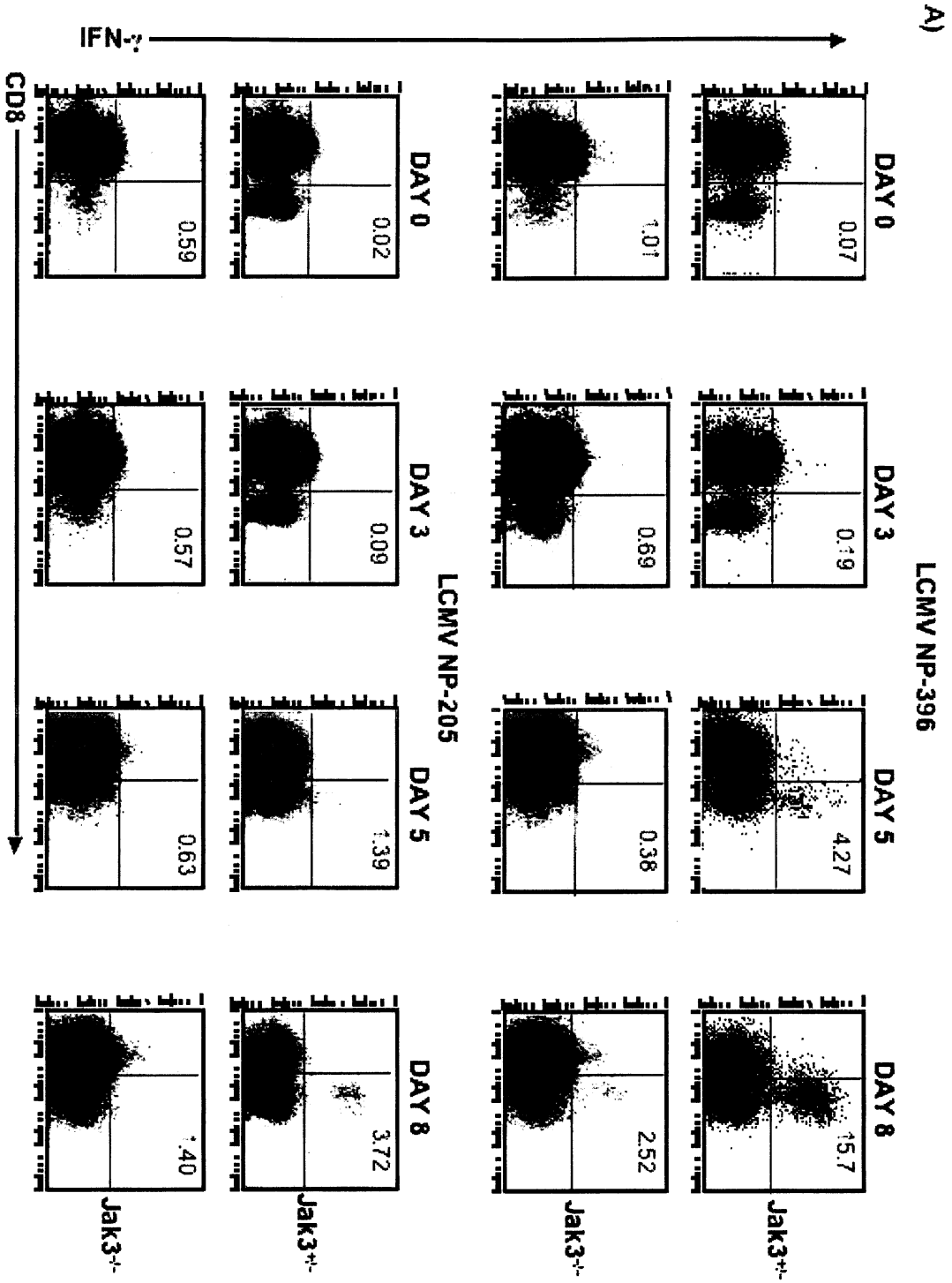

A)
DAY 0
DAY 3
DAY 5
DAY 8
10.3
9.7
7.7
37.7
14.8
16.3
7.2
10.3
Jak3+/-
1.94
2.7
2.9
2.1
22.1
34.8
16.8
28.6
Jak3-/-
CD8
CD4
B)
CD8 gated T cells
DAY 0
DAY 3
DAY 5
DAY 8
19.3
7.5
49.8
91.5
M1
Jak3+/-
69.8
56.3
72.8
86.4
Jak3-/-
CD44

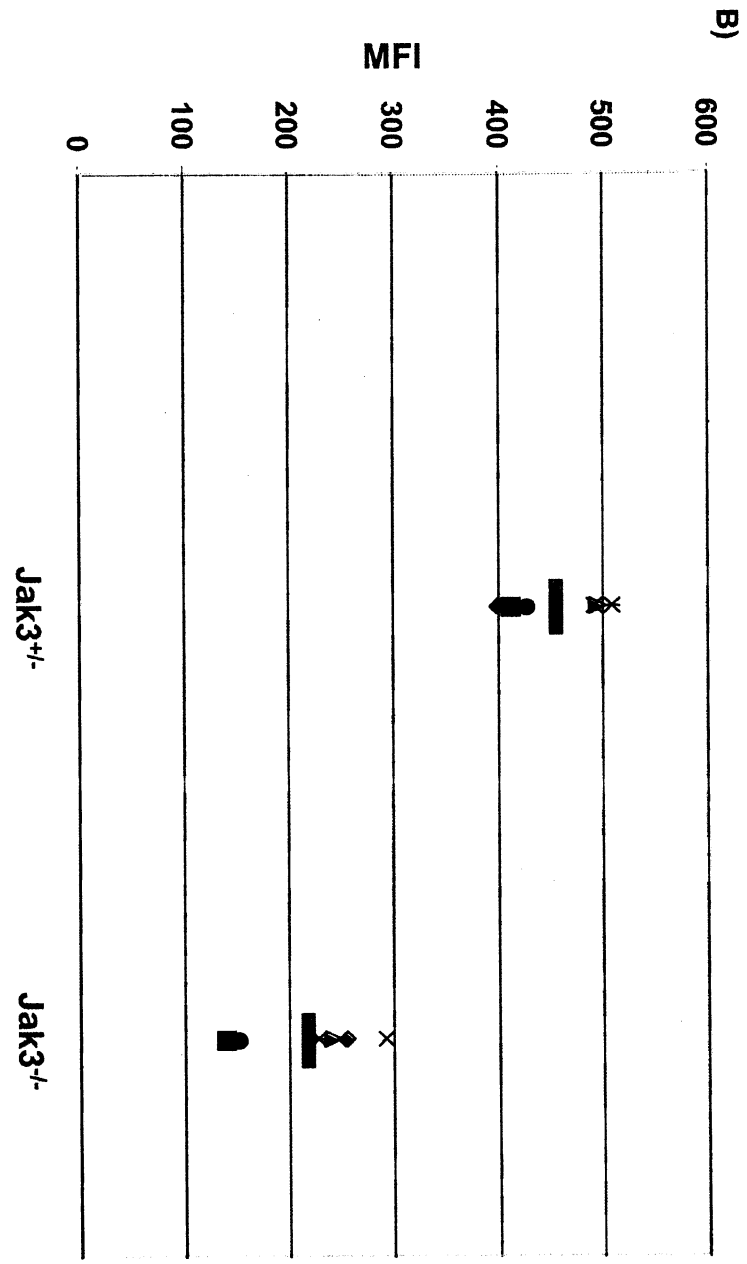
To address the functional capabilities of Jak3$^{-/-}$ CD8^{+} T cells, I examined IFN-γ production by CD8^{+} T cells in response to specific LCMV peptides. For these experiments, I used LCMV NP-396 and GP-33, both immunodominant peptides, as well as NP-205, a subdominant peptide, and stimulated freshly isolated splenocytes from LCMV-infected mice. Intracellular cytokine staining for IFN-γ, TNF-α and IL-2 was performed after a 5 hour *ex vivo* incubation. As shown in Figure 16 A, no significant cytokine response was observed before day 5 post-infection by Jak3$^{+/-}$ CD8^{+} T cells. At day 5, ~4% of the Jak3$^{+/-}$ CD8^{+} T cells produced IFN-γ in response to NP-396 and ~1.4% in response to NP-205. In contrast, Jak3$^{-/-}$ CD8^{+} T cells did not produce any detectable IFN-γ at this time point, even though there was a clear expansion in total CD8^{+} T cell numbers in these mice. By day 8 post-infection, ~15% of the Jak3$^{+/-}$ CD8^{+} T cells were producing IFN-γ in response to the dominant peptide and ~4% in response to the subdominant peptide. At this time point, only ~2.5% of Jak3$^{-/-}$ CD8^{+} T cells made IFN-γ in response to NP-396 and ~1.4% in response to NP-205. Furthermore, the amount of IFN-γ secreted by the Jak3-deficient CD8^{+} T cells at this time point was significantly reduced compared to the amount secreted by the WT cells, as determined by the mean fluorescent intensities (Fig.17 B). The CD8^{+} T cell responses to GP-33 stimulation were similar, if not greater than those obtained in response to NP-396 (data not shown). Jak3$^{+/-}$ CD8^{+} T cells produced small amounts of IL-2 at these time points, consistent with previously published data [270]. Similar to IFN-γ, the amount of IL-2 produced by Jak3$^{-/-}$ CD8^{+} T cells was inhibited compared to Jak3$^{+/-}$ control T cells (data not shown). In contrast to Jak3$^{+/-}$ T cells, Jak3$^{-/-}$ CD8^{+} cells did not produce TNF-α in response to any of

the peptides tested (Fig.17 C). This phenomenon has been observed in mice chronically infected with LCMV. $CD8^+$ T cells progressively lose their effector function in a hierarchical fashion [270]. The ability to produce TNF-α was one of the first functions to be compromised, along with IL-2 production and target lysis. IFN-γ production was most resistant to functional exhaustion in this system [270]. Finally, the ability of $CD8^+$ T cells to produce granzyme B was severely inhibited in $Jak3^{-/-}$ mice (data not shown), a finding consistent with impaired cytotoxicity.

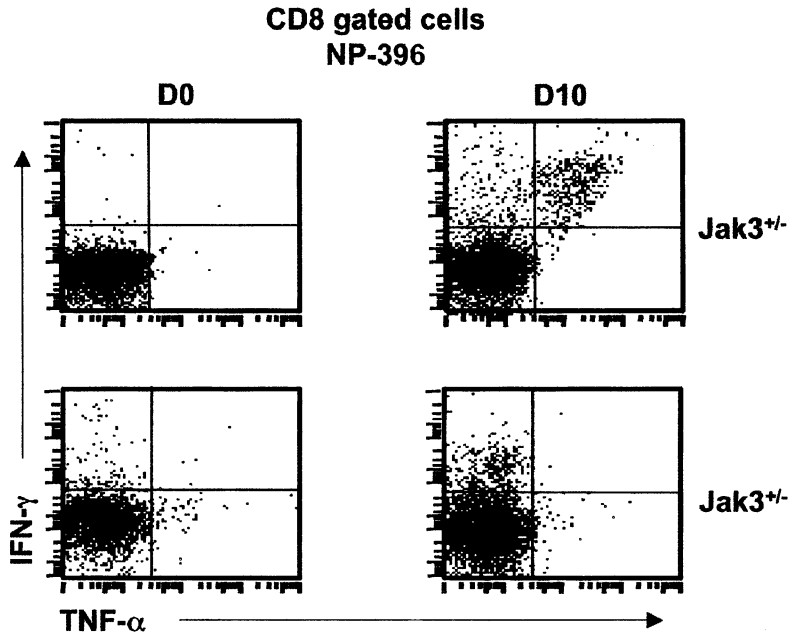
Fig.17 Jak3$^{-/-}$ CD8^{+} T cells produce low levels of IFN-γ and no TNF-α production in response to LCMV

Eight days after LCMV infection, total splenocytes from Jak3$^{-/-}$ and control Jak3$^{+/-}$ mice were restimulated for 5 hours *ex vivo* with 5μM synthetic peptides, LCMV NP396 (A), NP205, or GP33 (data not shown) at different timepoints following LCMV infection. Production of IFN-γ (A), TNF-α (C) and IL-2 (data not shown) by CD8^{+} T cells was measured by intracellular cytokine staining (materials and methods). Each sample was also stained with isotype control Abs. (data not shown). The numbers represent the percent of CD8^{+} T cells making IFN-γ (A) or TNF-α (C). B) The mean fluorescent intensities of IFN-γ production were measured for all samples and the average is depicted as a red line. The Jak3$^{-/-}$ results are representative of mice that were able to mount a CD8^{+} T cell-mediated effector response (~40% of all mice investigated). The Jak3$^{+/-}$ mice are representative of all mice investigated whose responses were always consistent.


A)
IFN-γ
CD8
LCMV NP-396
LCMV NP-205
DAY 0
DAY 3
DAY 5
DAY 8
0.07
0.19
4.27
15.7
1.01
0.69
0.38
2.52
0.02
0.09
1.39
3.72
0.59
0.57
0.63
1.40
Jak3+/-
Jak3+/-
Jak3-/-
Jak3-/-


B)
MFI
600
500
400
300
200
100
0
Jak3+/-
Jak3-/-

C)



To examine the effectiveness of the $CD8^+$ T cell response to LCMV, I investigated whether $Jak3^{-/-}$ mice could eliminate the virus from their spleens. As shown in Table II, $Jak3^{+/-}$ control mice have high titers of LCMV in their spleens at day 3 post-infection, but have eradicated the virus by day 8 following infection. In contrast, $Jak3^{-/-}$ mice are unable to mount a protective immune response to LCMV, and have increased titers of LCMV at day 8 compared to day 3 post-infection. Surprisingly, $Jak3^{-/-}$ mice presented lower viral titers during the early phase of the infection, suggesting that the ability of the virus to infect target cells was impaired in the Jak3-deficient environment. Type I interferons, IFN-α and -β, have been shown to inhibit unlimited viral replication upon LCMV infection [271]. Therefore, the decreased levels of viral titers observed in the $Jak3^{-/-}$ mice may be due to higher levels of type I interferons in these mice. This hypothesis was not investigated, but recent observations from Joonsoo Kang's group indicate that defects in the STAT 5 signaling pathway may lead to dysregulated signaling through STAT 1, which is required for type I interferon receptor signaling. I also examined spleens from mice one month after LCMV infection and observed that $Jak3^{-/-}$ mice still carried high titers of the virus. Thus, the $CD8^+$ T cell response observed in $Jak3^{-/-}$ mice is either not sufficiently potent, or not rapid enough to clear the LCMV from the periphery.

Table II Jak3-deficient mice are not able to clear an LCM virus infection

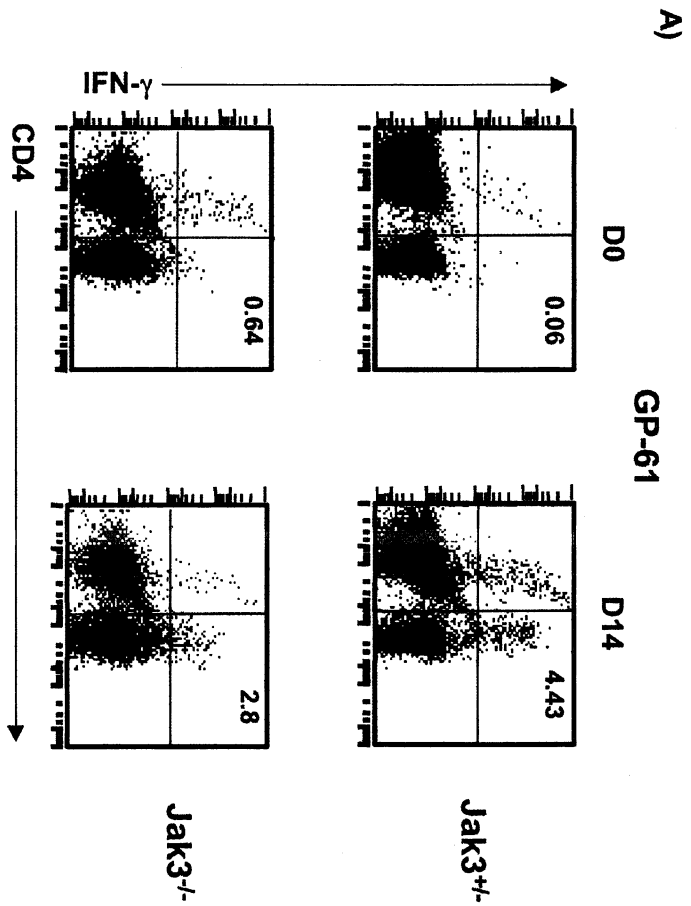
Viral titers were calculated at different time points following LCMV infection by plaque assay analysis of spleens from infected and uninfected mice, as described in the materials and methods. The viral titers for 2-4 $Jak3^{-/-}$ or $Jak3^{+/-}$ control mice, at each timepoint were calculated. The table shows the average of PFU/mL per spleen of each mouse genotype at the specific times following LCMV infection.

LCMV infection	Day 0	Day 3	Day 8	Day 30
$Jak3^{+/-}$	N/D	$4.5 \pm 0.7 \times 10^5$	N/D	N/D
$Jak3^{-/-}$	N/D	$0.8 \pm 0.7 \times 10^5$	$1.4 \pm 0.8 \times 10^6$	$3.5 \pm 3 \times 10^5$

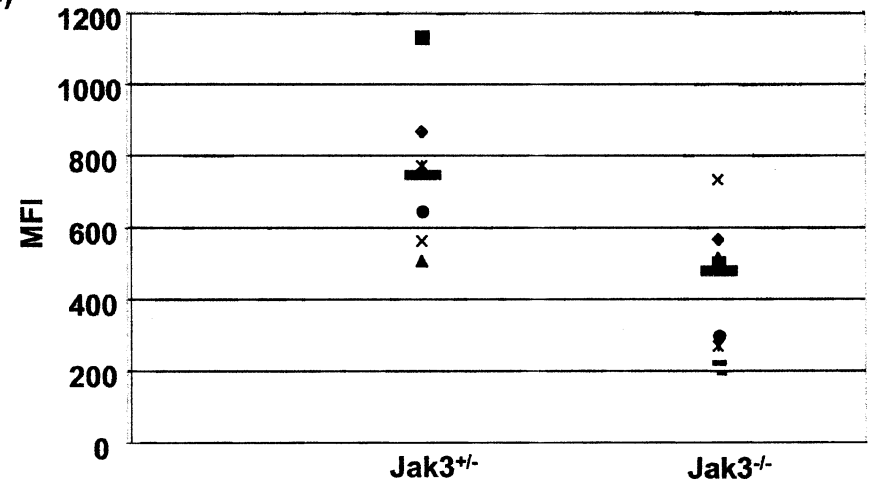
In an effort to determine why only a fraction of $Jak3^{-/-}$ mice (40%) made a $CD8^+$ T cell response to LCMV infection, albeit a limited one, I examined the capability of $CD4^+$ T cells to become activated in response to LCMV. Even though $CD8^+$ T cells from WT mice can mount a functional response in the absence of $CD4^+$ T cell help, I reasoned that $Jak3^{-/-}$ $CD8^+$ T cells may be more dependent on this help. As mentioned above, the percentage of $CD4^+$ T cells in Jak3-deficient mice are highly elevated compared to wild type control mice. During the course of the immune response, the percentage of $Jak3^{+/-}$ $CD4^+$ T cells remained fairly constant, with elevated total numbers at the peak of the response. Likewise, the percentages of $Jak3^{-/-}$ $CD4^+$ T cells remained constant, but in contrast to the WT control mice, the total numbers also remained unchanged. I examined IFN-γ and TNF-α production by $CD4^+$ T cells, using an MHC-class-II dominant epitope, GP-61. Between day 12 and day 14, the peak of the CD4 immune response, freshly isolated cells from infected $Jak3^{-/-}$ and $Jak3^{+/-}$ cells were restimulated with the epitope *in vitro*, for five hours. $CD4^+$ T cells from WT mice made a significant amount of IFN-γ (~4.5 %) (Fig.18 A). Most of these cells also produced TNF-α (Fig.18 B). $CD4^+$ T cells from Jak3-deficient mice were able to produce IFN-γ (~2.8%), but the percentage of responding cells, as well as the amount of cytokine produced were reduced compared to the control mice (Fig.18 A and B). Similarly to $CD8^+$ T cells, responding $CD4^+$ T cells did not produce TNF-α in response to LCMV (Fig.18 C).

Fig.18 Jak3$^{-/-}$ CD4^{+} T cells produce low levels of IFN-γ and no TNF-α in response to LCMV

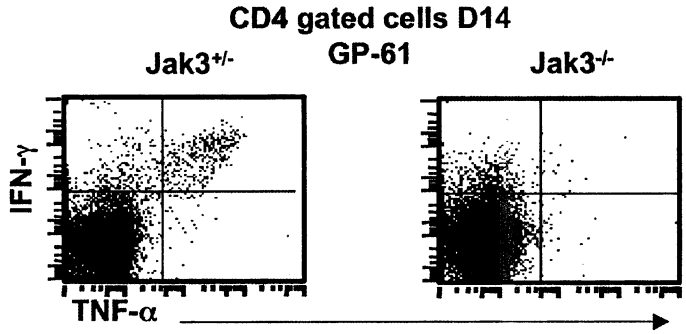
Total splenocytes from Jak3$^{-/-}$ and control Jak3$^{+/-}$ mice were restimulated for 5 hours *ex vivo* with 5μM synthetic peptide, LCMV GP61 at different timepoints following LCMV infection. Production of IFN-γ (A), TNF-α (C) and IL-2 (data not shown) by CD4^{+} T cells was measured by intracellular cytokine staining (materials and methods). Each sample was also stained with isotype control Abs. (data not shown). The numbers represent the percent of CD4^{+} T cells making IFN-γ (A) or TNF-α (C). B) The mean fluorescent intensities of IFN-γ production were measured for all samples and the average is depicted as a red line. The Jak3$^{-/-}$ results are representative of mice that were able to mount a CD4^{+} T cell-mediated effector response (~80% of all mice investigated, n=12). The Jak3$^{+/-}$ mice are representative of all mice investigated whose responses where always consistent.


A)
IFN-γ
CD4
D0
D14
GP-61
0.06
4.43
0.64
2.8
Jak3+/-
Jak3-/-

B)


1200
1000
800
600
400
200
0
MFI
Jak3+/-
Jak3-/-

C)


CD4 gated cells D14
GP-61
Jak3+/-
Jak3-/-
IFN-γ
TNF-α

B.2 Constitutive Bcl-2 expression restores the naïve $CD8^+$ T cell compartment in OT-1 TCR transgenic $Jak3^{-/-}$ mice

One concern about the experiments described above is the possibility that the poor anti-viral response by $Jak3^{-/-}$ $CD8^+$ T cells is due to a greatly reduced TCR repertoire in $Jak3^{-/-}$ mice. For instance, our previous studies have shown that peripheral $CD4^+$ T cells in $Jak3^{-/-}$ mice have a highly skewed TCR repertoire not representative of the diversity of receptor specificities found in wild type mice [265]. Should this be the case, $Jak3^{-/-}$ mice might display an impaired anti-viral response due to greatly reduced numbers of LCMV-specific $CD8^+$ T cells, and not because the cells are functionally impaired.

To circumvent this concern, I generated mice with normal numbers of peripheral $CD8^+$ T cells in the absence of Jak3. As described in chapter IV, by crossing $Jak3^{-/-}$bcl-2 transgenic mice to the OT-1 TCR transgenic line I was able to generate mice that had large numbers of peripheral $CD8^+$ T cells expressing the OT-1 TCR (Fig.15). In fact, $Jak3^{-/-}$bcl-2 transgenic OT-1^+ mice had comparable numbers of peripheral $CD8^+$ T cells as control $Jak3^{+/-}$ OT-1^+ mice; furthermore, the $V\alpha2^+$ $CD8^+$ T cells in all of these mice were virtually all $CD44^{lo}$.

B.3 $Jak3^{-/-}$OT-1^+bcl-2 transgenic $CD8^+$ T cells are activated efficiently *in vitro*, but proliferate poorly

The naive $CD8^+$ T cells from these $Jak3^{-/-}$OT-1^+bcl-2 transgenic mice provided an opportunity to assess T cell survival, activation, proliferation, and cytokine production responses for $CD8^+$ T cells lacking all γ_c/Jak3-dependent cytokine signals. In a first

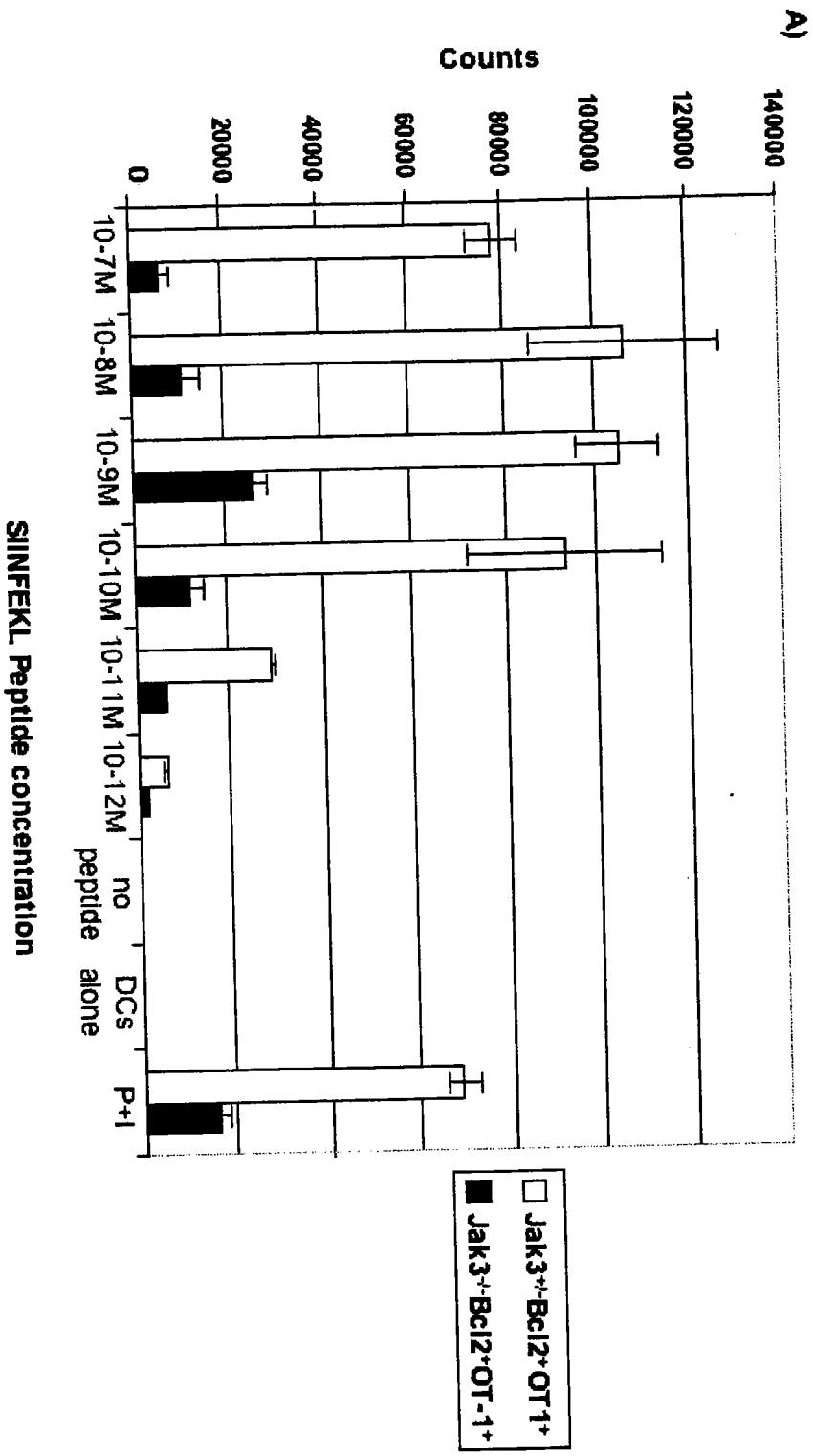
series of experiments, I examined T cell responses *in vitro*, following stimulation of purified $CD8^+$ T cells from $Jak3^{-/-}OT\text{-}1^+bcl\text{-}2^+$ or $Jak3^{+/-}OT\text{-}1^+bcl\text{-}2^+$ control mice with dendritic cells (DCs) loaded with the ovalbumin peptide, SIINFEKL. After 48 hours, cells were pulsed with 3H-thymidine, and proliferative responses were assessed. As shown in Figure 19 A, $CD8^+$ T cells from $Jak3^{-/-}bcl\text{-}2^+OT\text{-}1^+$ mice did proliferate in response to the ovalbumin peptide with a dose response similar to the control $Jak3^{+/-}bcl\text{-}2^+OT\text{-}1^+$ cells. However, the magnitude of the proliferative response by the $Jak3^{-/-}$ T cells was greatly reduced, and only reached a maximum of ~25% that of the controls. A comparable response was also observed to the pharmacological agents, PMA plus ionomycin. Nonetheless, these data demonstrate the remarkable finding that naïve $CD8^+$ T cells completely lacking all γ_c/Jak3-dependent cytokine signals can mount a modest proliferative response. This finding is in direct contrast to that seen with peripheral T cells from non-transgenic $Jak3^{-/-}$ mice, which are completely non-responsive to mitogenic stimulation *in vitro*.

I also assessed the activation status of $Jak3^{-/-}bcl\text{-}2^+OT\text{-}1^+$ $CD8^+$ T cells stimulated *in vitro* with antigen plus APCs. 48 hours following stimulation, the cells were isolated and stained with antibodies to CD8 and CD44. Figure 19 B shows the percentage of $CD44^{hi}$ $CD8^+$ T cells from $Jak3^{-/-}bcl\text{-}2^+OT\text{-}1^+$ and control $Jak3^{+/-}bcl\text{-}2^+OT\text{-}1^+$ mice. Interestingly, the $Jak3^{-/-}$ cells were activated as efficiently as the control $CD8^+$ T cells, and showed a comparable peptide dose response curve. These data indicate that naïve $CD8^+$ T cells can be activated in the absence of all γ_c/Jak3-dependent cytokine signals.

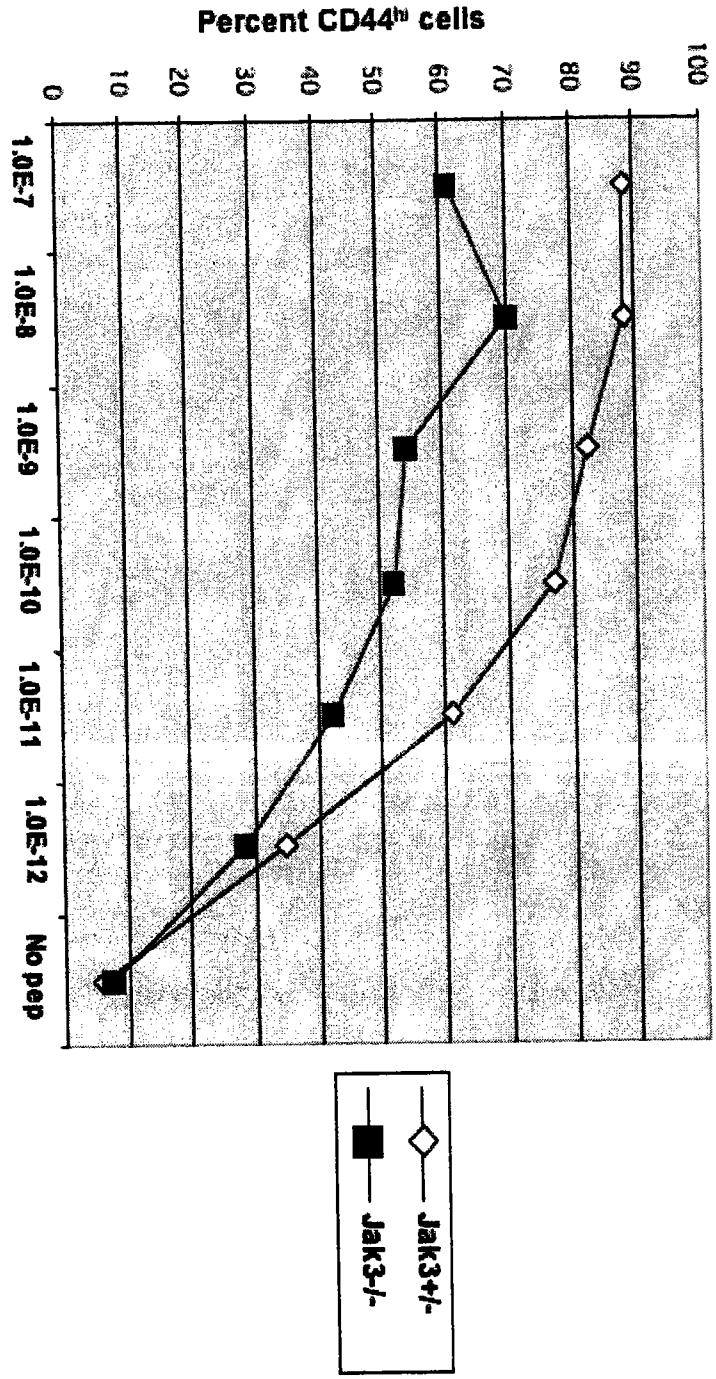
The discrepancy between the proliferative response and the activation status of $Jak3^{-/-}$bcl-2^{+}OT-1^{+} $CD8^{+}$ T cells following *in vitro* stimulation prompted me to examine the survival properties of these cells following *in vitro* culture. In spite of the constitutive expression of Bcl-2, $Jak3^{-/-}$ T cells show markedly reduced survival after 48 hours in culture compared to control T cells (Fig.19 C). This impaired survival was observed regardless of the peptide concentration used to stimulate the T cells, and was independent of the extent of T cell proliferation seen at each condition.

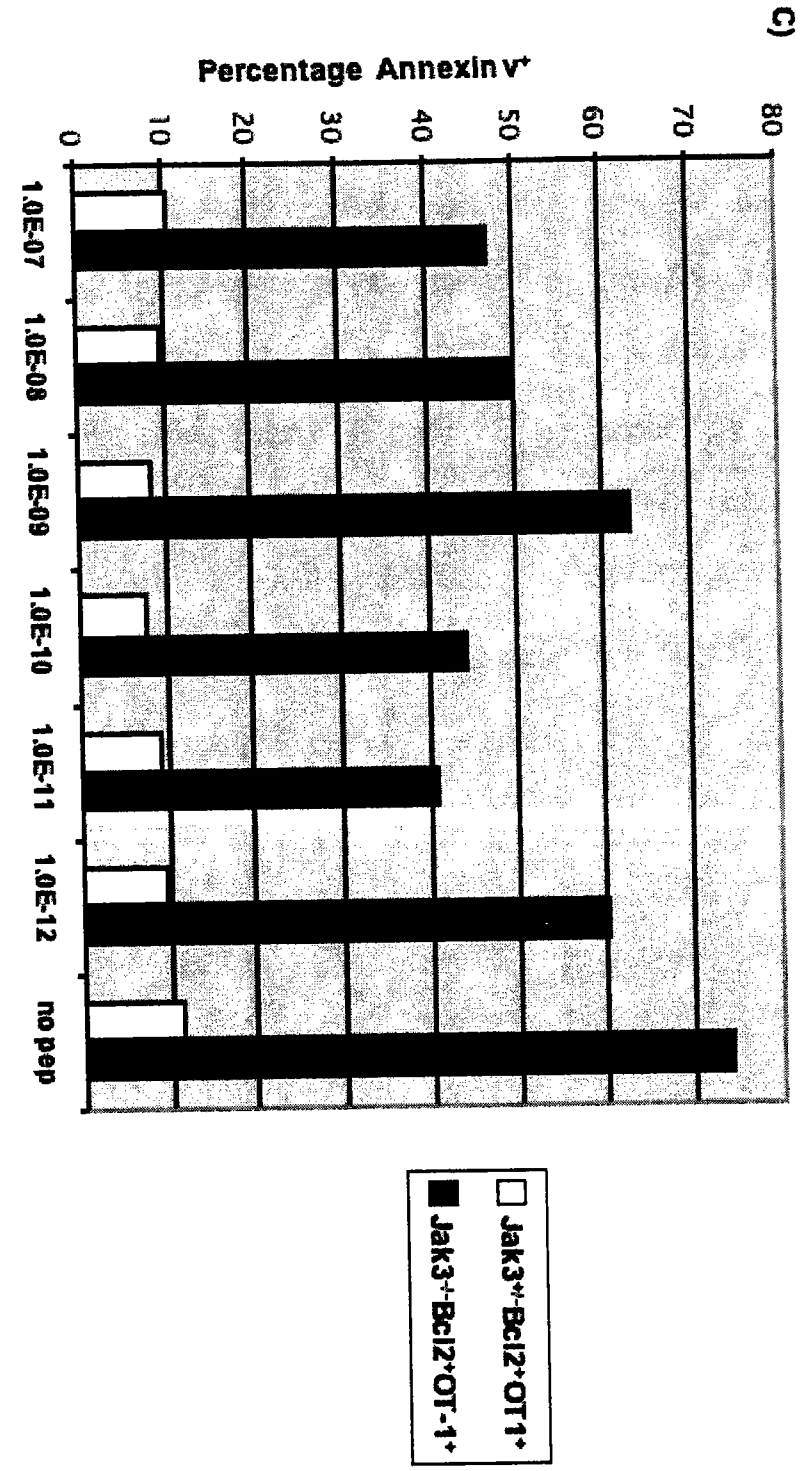
Fig.19 $CD8^+$ T cell activation results in reduced proliferation and increased apoptosis in the absence of Jak3/γ_c-mediated signals

Using bone marrow derived dendritic cells pulsed with different concentrations of SIINFEKL peptide, the same number of $CD8^+OT\text{-}1^+bcl\text{-}2^+$ cells from $Jak3^{-/-}$ or $Jak3^{+/-}$ control mice were stimulated *in vitro* for 48 hours. As controls, the same number of $CD8^+OT\text{-}1^+bcl\text{-}2^+$ T cells from each mouse genotype was stimulated in the presence of non-pulsed DCs. A) 24 hours post stimulation 1μCi/well ^{3}[H]-thymidine was added to some of the cultures and the amount of $CD8^+$ T cell proliferation was determined by thymidine incorporation ~20 hours later (~48 hour incubation total). B) The activation status of cultured $CD8^+$ T cells was determined by CD44 and CD62-L (data not shown) expression. C) The percentage of cells undergoing apoptosis was determined by staining cells with PI and annexin V.


A)
Counts
140000
120000
100000
80000
60000
40000
20000
0
10-7M
10-8M
10-9M
10-10M
10-11M
10-12M
no peptide
DCs alone
P+I
SIINFEKL Peptide concentration
Jak3+/-Bcl2+OT1+
Jak3-/-Bcl2+OT-1+

B)

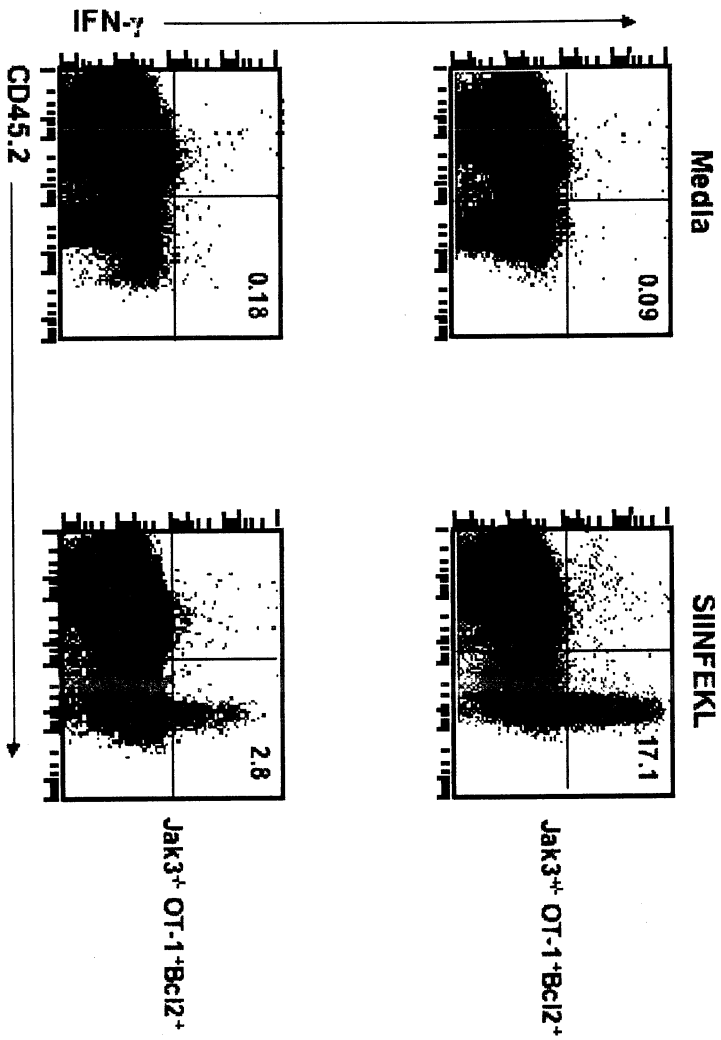

Percent CD44hi cells
0
10
20
30
40
50
60
70
80
90
100
1.0E-7
1.0E-8
1.0E-9
1.0E-10
1.0E-11
1.0E-12
No pep
Jak3+/-
Jak3-/-


C)
Percentage Annexin v+
0
10
20
30
40
50
60
70
80
1.0E-07
1.0E-08
1.0E-09
1.0E-10
1.0E-11
1.0E-12
no pep
Jak3+/+Bcl2+OT1+
Jak3-/-Bcl2+OT-1+

Finally, I investigated the ability of Jak3-deficient OT-1^{+}bcl-2^{+} T cells to make IFN-γ in response to antigenic stimulation *in vitro*. Positively selected CD8^{+} T cells from Jak3$^{-/-}$ or Jak3$^{+/-}$OT-1^{+}bcl-2^{+} mice were incubated in the presence of SIINFEKL-pulsed congenic WT splenocytes for 48 hours. Golgi plug was added during the last five hours of the incubation, and IFN-γ production was measured by intracellular cytokine staining. A moderate percentage of the Jak3$^{+/-}$ control CD8^{+} T cells was able to make significant amounts of IFN-γ by this time point (~12% of the CD8^{+} T cells) (Fig.20). In contrast, only a very limited percentage of the Jak3$^{-/-}$ CD8^{+} T cells in these cultures was able to produce IFN-γ in response to the peptide stimulation, and the percentage of Jak3$^{-/-}$ CD8^{+} T cells remaining in the culture were reduced compared to the Jak3$^{+/-}$ control cells (Fig.20).

Fig.20 Reduced levels of $CD8^+$ IFN-γ effector cells and IFN-γ production *in vitro*, in the absence of Jak3

The same number of $V\alpha2^+$ $CD8^+$ T cells from $Jak3^{+/-}$ or $Jak3^{-/-}$bcl-2^+OT-1^+ mice were stimulated *in vitro* by SIINFEKL-pulsed congenic splenocytes. As a negative control, the same number of Vα2 $CD8^+$ T cells from each genotype were incubated in the presence of congenic splenocytes that had not been pulsed with the peptide. Golgi plug was added to all the wells after 40 hours of incubation at 37°. Five hours later, the cells were stained for surface markers (CD4, CD8, CD45.2) and then intracellularly stained for IFN-γ or an isotype control Ab. The left panels depict the $Jak3^{+/-}$ $CD8^+$ T cells, the right panels depict $Jak3^{-/-}$ $CD8^+$ T cells that were incubated in the presence of splenocytes alone (top) or SIINFEKL-pulsed splenocytes (bottom).


IFN-γ
CD45.2
Media
SIINFEKL
0.09
0.18
17.1
2.8
Jak3+/+ OT-1+Bcl2+
Jak3+ OT-1+Bcl2+

B.4 Adoptively-transferred Jak3$^{-/-}$bcl-2^{+}OT-1^{+} CD8^{+} T cells expand and differentiate in response to viral infection

The presence of large numbers of naïve CD8^{+} T cells of known antigenic specificity in Jak3$^{-/-}$bcl-2^{+}OT-1^{+} mice also provided an opportunity to assess the *in vivo* capabilities of these T cells. To utilize a system as physiologically-relevant as possible, I took advantage of vaccinia virus recombinants that had been engineered to express the chicken ovalbumin protein (vaccinia-ova). Thus, after infection of mice, virus-infected cells will express and present the ovalbumin antigen containing the OT-1-specific epitope (SIINFEKL). To assess the responsiveness of Jak3$^{-/-}$ CD8^{+} T cells, I used an adoptive transfer protocol, where 2.5 x 10^{6} purified CD8^{+} T cells (see materials and methods) from Jak3$^{-/-}$bcl-2^{+}OT-1^{+} or Jak3$^{+/-}$bcl-2^{+}OT-1^{+} mice were introduced into congenic (C57BL/6-CD45.1^{+}) recipients one day prior to vaccinia-ova infection. This strategy provided an important advantage, in that CD4^{+} and CD8^{+} T cells in the host would be present to generate a wild type immune response to vaccinia virus, thus providing any CD4^{+} T cell help or other important accessory functions.

Following adoptive transfer and infection with vaccinia-ova, the activation, expansion, and differentiation of the transferred OT-1^{+} CD8^{+} T cells was assessed at days 2, 5, and 8 post-infection (Fig.21). Day 0 represents the mice that were injected with cells but not infected with the vaccinia-ova construct. These mice were analyzed 72 hours following adoptive transfer of the OT-1^{+} T cells. At this timepoint, a very small population of OT-1^{+} T cells (CD45.2^{+}) are visible in mice receiving Jak3$^{+/-}$bcl-2^{+}OT-1^{+} cells (Fig.21 A). These cells are predominantly CD44^{lo}, as expected (Fig.21 B). The

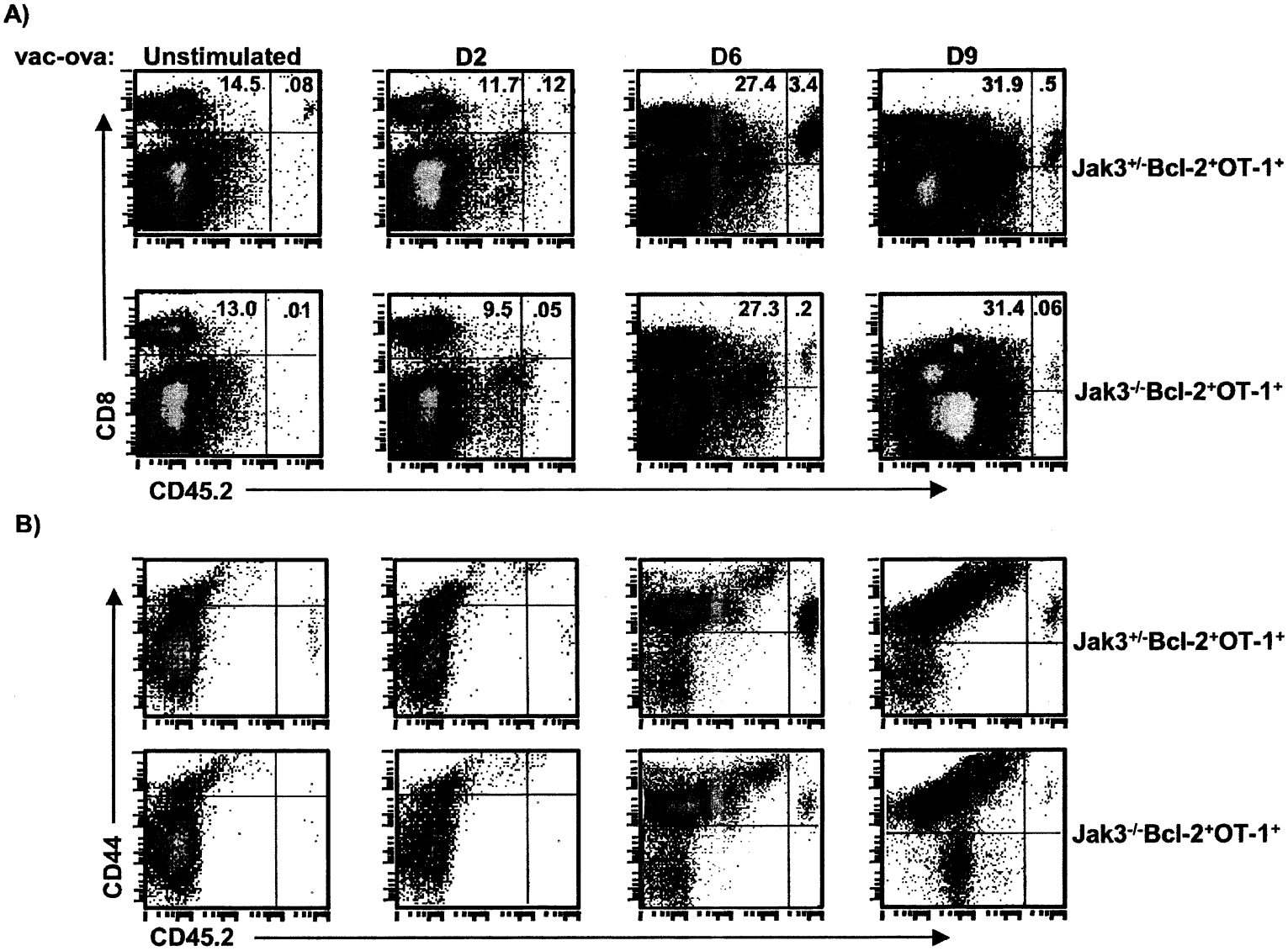
transferred Jak3$^{-/-}$bcl-2^{+}OT-1^{+} cells are more difficult to detect at this timepoint, perhaps due to their diminished survival properties. Following vaccinia-ova infection, a response by the transferred T cells is first visible at day 6. In mice receiving Jak3$^{+/-}$bcl-2^{+}OT-1^{+} cells, the OT-1^{+} T cells represent >3% of splenocytes at day 6 post-infection, a 30-40-fold expansion from the starting population. Expansion of the transferred Jak3$^{-/-}$bcl-2^{+}OT-1^{+} cell population was also visible at this timepoint, although the proportion of Jak3$^{-/-}$ T cells was substantially less than that for the control cells. As expected, both Jak3$^{+/-}$ and Jak3$^{-/-}$ populations of expanded OT-1^{+} cells were CD44^{hi} at day 6. Given the reduced numbers of CD8^{+} Jak3$^{-/-}$ T cells still present 3 days post cell transfer, it is possible that the decreased accumulation of activated Jak3$^{-/-}$ CD8^{+} T cells observed at the peak of the response is due to the limited starting Jak3$^{-/-}$ T cell population, and not to an inability of these cells to proliferate. By day 9 post-infection, the OT-1^{+} CD8^{+} T cells in both sets of animals were reduced in percentage compared to the peak at day 6, and the cells remaining were all CD44^{hi}.

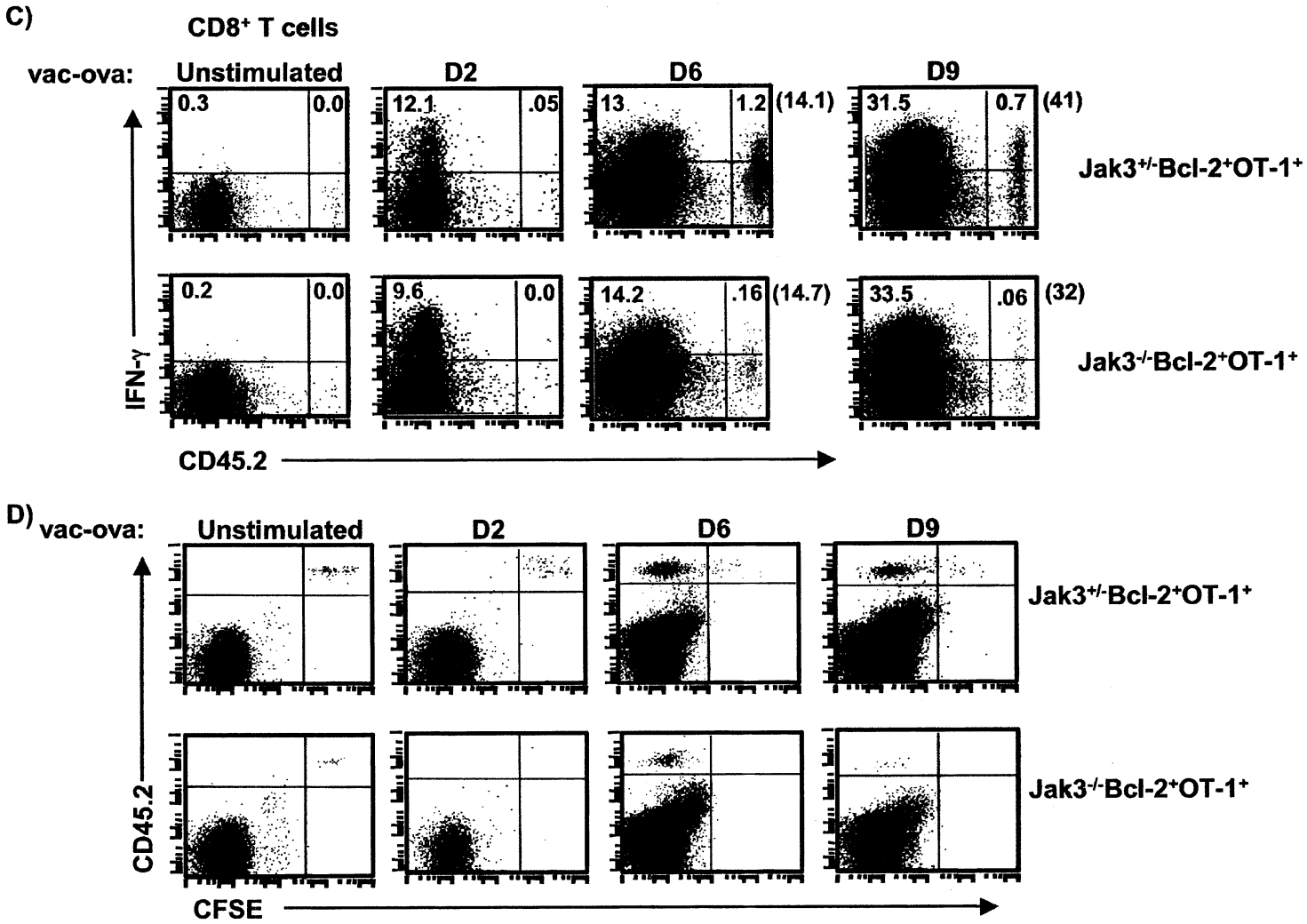
To assess the differentiation status of the activated OT-1^{+} CD8^{+} T cells in the vaccinia-ova-infected mice, I examined their ability to produce IFN-γ upon *ex vivo* stimulation with PMA plus ionomycin. As shown in Figure 21 C, a comparable proportion of the transferred Jak3$^{+/-}$ and Jak3$^{-/-}$ T cells are capable of producing IFN-γ at days 6 and 9 post-infection. As another measure of the functional response of the transferred T cells to the virus infection, I also examined proliferation *in vivo*. For this analysis, the purified CD8^{+} T cells from the Jak3$^{-/-}$bcl-2^{+}OT-1^{+} and the Jak3$^{+/-}$bcl-2^{+}OT-1^{+} mice were labeled with the fluorescent dye, CFSE, prior to adoptive transfer. As can

be seen in Fig.21 D, the transferred cells, wherever visible, remain CFSE-high (i.e., undivided) at day 0 and day 2 post-infection. By day 6 post-infection, all detectable transferred OT-1^+ T cells are completely CFSE-negative. These findings indicate that the T cell proliferation is occurring between day 2 and day 5 post-infection, and that both the $Jak3^{-/-}$ and the $Jak3^{+/-}$ cells are undergoing maximum rounds of cell division. Nonetheless, transfer of the identical number of OT-1^+ T cells from the two donor strains results in a substantially reduced accumulation of $Jak3^{-/-}$ $CD8^+$ T cells. This conclusion indicates a profound defect in survival by activated $Jak3^{-/-}$ $CD8^+$ T cells, even in the presence of high levels of the pro-survival factor, Bcl-2.

Fig.21) Reduced accumulation of Jak3$^{-/-}$ CD8^{+} T cells during an *in vivo* immune response

2.5 x 10^6 CFSE-labelled CD8^{+}T cells from Jak3$^{+/-}$ or Jak3$^{-/-}$ OT-1^{+}bcl-2^{+} mice were injected i.v. into congenic (CD45.1) mice. 24 hours later all mice, except for the uninfected control mice, were injected with 1.0x10^7 PFUs of a vaccinia-ova construct. At day 3 post cell-transfer and day 2 post vaccinia-ova injection the spleens from uninfected and 2-day infected mice were isolated. The ability of transferred cells to survive, proliferate and respond to the antigenic challenge was analyzed. Similarly, 6 and 9 days following the vaccinia-ova injection, spleens from infected mice were isolated and the transferred cells investigated. A) The percentage of CD8^{+} transferred T cells was calculated by CD45.2 mAb staining, at the different timepoints. B) Their activation status was analyzed by gating on CD8^{+} T cells and analyzing CD44 expression. C) Their ability to differentiate into effector cells was investigated by analyzing the production of IFN-γ following ex vivo restimulation for five hours. D) The ability of transferred CD8^{+} T cells to proliferate was investigated by examining the amount of CFSE dilution.


A)
vac-ova:
Unstimulated
D2
D6
D9
14.5
.08
11.7
.12
27.4
3.4
31.9
.5
Jak3+/-Bcl-2+OT-1+
13.0
.01
9.5
.05
27.3
.2
31.4
.06
Jak3-/-Bcl-2+OT-1+
CD8
CD45.2
B)
Jak3+/-Bcl-2+OT-1+
Jak3-/-Bcl-2+OT-1+
CD44
CD45.2


C)
CD8+ T cells
vac-ova:
Unstimulated
D2
D6
D9
0.3
0.0
12.1
.05
13
1.2
(14.1)
31.5
0.7
(41)
Jak3+/-Bcl-2+OT-1+
0.2
0.0
9.6
0.0
14.2
.16
(14.7)
33.5
.06
(32)
Jak3-/-Bcl-2+OT-1+
IFN-γ
CD45.2
D)
vac-ova:
Unstimulated
D2
D6
D9
Jak3+/-Bcl-2+OT-1+
Jak3-/-Bcl-2+OT-1+
CD45.2
CFSE

C. DISCUSSION

Cytokines of the γ_c/Jak3-dependent family are known to play a critical role in the maintenance of T cell homeostasis. IL-7 is important for T cell development, as well as for the survival of naïve $CD4^+$ and $CD8^+$ T cells, while IL-15 is involved in the proliferation and thus in the maintenance of memory $CD8^+$ T cells. The role of IL-2 has been more difficult to establish, as various studies have demonstrated defects in both the proliferation of $CD4^+$ and $CD8^+$ T cells, as well as in activation induced cell death in the absence of IL-2 receptor signaling. Given these diverse and crucial roles for γ_c/Jak3-dependent cytokines, together with the knowledge that γ_c- or Jak3-deficient humans have a fatal severe combined immunodeficiency disease (SCID), it was always assumed that these cytokine signals would be essential for all T cell function.

It has been particularly difficult to address the role of γ_c/Jak3-dependent cytokine signals for $CD8^+$ T cell function, as mice lacking these cytokine signals have severe deficiencies in peripheral $CD8^+$T cell numbers. A previous study was successful in examining the antigen-specific responsiveness of γ_c-deficient $CD4^+$ T cells, and concluded, surprisingly, that these T cells were nearly normal for proliferation, differentiation, and effector cytokine production following *in vivo* activation. However, no comparable study has yet been performed on γ_c- or Jak3-deficient $CD8^+$ T cells.

I examined the ability of $Jak3^{-/-}$ $CD8^+$ T cells to respond to viral infections using two independent systems. The first utilized intact $Jak3^{-/-}$ mice infected with LCMV, and took advantage of the immense magnitude of the $CD8^+$ T cell response to this virus, in addition to the wealth of useful reagents for characterizing LCMV-specific T cell

responses. As a second system, I utilized TCR transgenic Jak3$^{-/-}$ naïve CD8^{+} T cells expressing a receptor for chicken ovalbumin, and stabilized this cell population with constitutive expression of the Bcl-2 gene. These ovalbumin-specific T cells were then adoptively-transferred into wild type hosts, which were then infected with a recombinant vaccinia virus expressing chicken ovalbumin. An advantage of this latter system is that the T cell population under study is much more comparable between Jak3$^{-/-}$ and Jak3$^{+/-}$, and in addition, the transferred cells are responding to the virus in the context of an intact wild type host immune system.

In spite of the differences inherent in the two systems, both in the nature of the pathogens and in the differences in the responding cell populations, the results I obtained were generally consistent. However, I did observe a lack of consistency in the ability of intact Jak3$^{-/-}$ mice to respond to LCMV infections. Specifically, only 40% of the mice I examined were able to mount a CD8^{+} effector T cell response to LCMV, as measured by proliferation and IFN-γ production. Since Jak3$^{-/-}$ mice have extremely reduced numbers of mature CD8^{+} T cells, and their TCR repertoire is likely to be skewed, it is possible that the inability of some of these mice to respond to LCMV results from a low proportion or total absence of LCMV specific T cell clones in these individuals. Further, the response generated by the Jak3$^{-/-}$ mice that could produce an LCMV-specific CD8^{+} T cell effector population was extremely impaired, and ultimately, insufficient to clear the virus. Since, in this system it is impossible to track the starting population of LCMV-specific precursor cells in mice prior to infection, it is difficult to determine the relative expansion of virus-specific CD8^{+} T cells in Jak3$^{-/-}$ mice versus controls. Finally, mice deficient in

Jak3/γ_c-mediated signals lack NK cells and have reduced levels of $CD8^+$ DCs (Wallace, ME, unpublished data). Therefore, the activation of the innate arm of the immune system and consequent activation of the adaptive system may be impaired in these mice, which may result in the inability of $CD8^+$ T cells to become functional CTLs. Thus, the precise nature of the defect in generating a protective $CD8^+$ T cell response to LCMV in $Jak3^{-/-}$ mice cannot be deduced from these experiments.

For this reason, I turned to a second system where it was possible to ensure comparable numbers and surface phenotype (i.e., $CD44^{lo}$) of the starting populations of antigen-specific $CD8^+$ T cells. Using the OT-1 adoptive transfer system, I was able to carefully assess the survival, activation, expansion, and differentiation of $Jak3^{-/-}$ $CD8^+$ T cells while they were activated in a WT environment. These experiments demonstrated that, on a per cell basis, $Jak3^{-/-}$ $CD8^+$ T cells became activated, proliferated, and differentiated into cytokine-producing effector cells with comparable efficiency to control $CD8^+$ T cells in response to antigenic stimulation *in vivo*. In spite of this, the overall accumulation of antigen-specific $Jak3^{-/-}$ $CD8^+$ T cells was markedly reduced, averaging only ~10% compared to $Jak3^{+/-}$ cells. This striking defect is particularly significant, as identical numbers of OT-1^+ $CD8^+$ T cells were transferred from $Jak3^{-/-}$ and $Jak3^{+/-}$ donors; thus, in this case, the issue of precursor frequencies and/or absolute cell numbers prior to infection cannot be a factor. In general, these results are in agreement with recent reports that suggest that IL-2- and IL-15-deficient $CD8^+$ T cells are activated and can proliferate in response to a specific Ag but they cannot undergo the subsequent proliferative expansion and accumulation observed in WT mice by the peak of the

immune response [211, 212]. Our results suggest that the reduction in $CD8^+$ T cell responses is greater in the absence of all γ_c cytokine signals, probably due to the absence of cytokines that may, in previous studies, have provided compensatory functions. For example, recent reports have shown that IL-21 can enhance *in vitro* T cell responses to alloantigen during a primary stimulation, resulting in the generation of more potent effector T cells [272]. However, since the levels of $Jak3^{-/-}$ $CD8^+$ T cells in uninfected mice were significantly reduced 3 days after their adoptive transfers, it is also possible that $CD8^+$ T cell proliferation was not as impaired but that the $Jak3^{-/-}$ T cells were not able to survive and thus become activated and proliferate in response to the antigenic challenge, which was inflicted 24 hours after the transfer of the cells into the congenic hosts.

A second striking aspect of these findings is the fact that $Jak3^{-/-}$ $CD8^+$ T cells fail to survive and accumulate in spite of their constitutive expression of Bcl-2. It is unlikely that constitutive expression of Bcl-2 altered the functional responsiveness of the $CD8^+$ T cells in our experiments. Previous studies have shown that enforced expression of Bcl-2 leads to normal activation and subsequent deletion of activated T cells [117]. Instead, these data strongly suggest that cytokine signals are responsible for multiple aspects of cell survival that is independent of Bcl-2 up-regulation. It is also possible that, in the presence of competing $Jak3^{+/+}$ T cells in a WT environment, $Jak3^{-/-}$ $CD8^+$ T cells were more prone to apoptosis due to the lack of survival signals mediated through the IL-7 and IL-15 receptors. A comparison of the gene expression profiles of $Jak3^{-/-}CD8^+$bcl-2

transgenic versus Jak3$^{+/-}$CD8^{+}bcl-2 transgenic cells may provide information on the identity of these additional factors.

The results from our *in vitro* stimulation experiments with CD8^{+} Jak3$^{-/-}$OT-1^{+}bcl-2^{+} T cells were highly consistent with the data obtained from the *in vivo* infection models. Across the entire peptide dose-response tested, the proliferative response of Jak3$^{-/-}$ T cells was clearly detectable, but was markedly reduced compared to control cells. The fact that the Jak3$^{-/-}$ T cells proliferate at all following antigen stimulation is quite surprising, as these cells are incapable of responding to any of the known T cell growth factors. One possibility is that a few rounds of cell division following TCR/CD28 stimulation is cytokine-independent, as has previously been reported (JI170). In this study, however, CD8^{+} T cells underwent 3-4 rounds of division without requiring cytokine signals, but then failed to differentiate into cytokine-producing effector cells. In our hands, *in vitro* TCR stimulation of Jak3$^{-/-}$ CD8^{+} T cells was able to induce differentiation of IFN-γ-producing cells, however the numbers of responding T cells were reduced and the amount of cytokine produced was also severely impaired compared to WT CD8^{+} T cells.

Overall, our results suggest that CD8^{+} T cells can become activated and are able to undergo several rounds of proliferation, while differentiating into IFN-γ producing cells in the absence of γ_c cytokine signals. However, their ability to expand and mount a strong effector functional response is highly impaired. In the absence of Jak3/γ_c-mediated signals, CD8^{+} T cells lose their effector functionality, such as their ability to produce TNF-α or granzyme B, they are more susceptible to apoptosis than WT cells, and their response is not sufficient to clear a viral infection.

CHAPTER VI

DISCUSSION

From the earliest stages of development and throughout life, a properly functioning immune system is essential for the health and survival of all mammals. Failure of the immune system to protect the organism from infection, and inappropriate reactions of the system against the organism's own tissues result in chronic disease, immunodeficiencies, autoimmunity, and even death. Understanding the factors that regulate the proper function of the immune system is essential for the development of the necessary tools that will prevent and cure diseases and immune related disorders.

The development of adaptive immune responses is critical for the successful clearance of most pathogenic infections. The effector cells of the adaptive immune system, B and T lymphocytes are characterized by the expression of a highly diverse antigen receptor repertoire capable of recognizing virtually any foreign pathogen [1, 2, 4, 6, 7]. This diversity is generated through the random recombination of large families of antigen receptors genes during lymphocyte development. However, this diversity must be also tempered by the need to distinguish between self and non-self, to avoid inappropriate immune reactions against self-tissues. The ability of lymphocytes to do this is one of the most fascinating aspects of the immune system and complex regulatory mechanisms are required to establish and maintain this state [19, 20, 24, 28, 29]. Every stage of lymphocyte development, survival and function is therefore regulated not only by whether a lymphocyte expresses an appropriate antigen receptor, but also by the context in which it recognizes that antigen. More specifically, by the presence of self-MHC and costimulatory molecules, and by the cytokine milieu in which it receives these signals [66, 71-74, 92-95, 109, 110, 112, 196, 197, 202].

For T lymphocytes, cytokines that signals through the γ_c chain, play a prominent role in regulating almost every phase of their life cycle; from their development in the thymus, through steady state survival of mature lymphocytes, to their capacity to circulate throughout the body in search of infecting agents, and finally in the initiation and resolution of specific immune responses [74, 75, 179-181, 183, 185, 189, 196, 197, 200-203, 206-208, 210-212].

Signaling through γ_c depends uniquely on the Jak3 kinase [150, 151, 177-181]. A great deal of our understanding of the role of these cytokines in T cell immunity comes from analysis of Jak3-deficient mice. This chapter discuses the insights these studies have provided into the importance of the γ_c chain cytokines in T cell development, T cell homeostasis and T cell function.

T cell development takes place in the thymus where stem cells from the bone marrow commit to the T cell lineage in response to signals from the thymic environment [7]. In the absence of γ_c- or Jak3-mediated signals overall thymus cellularity is greatly reduced [16, 17, 179-181], and there is a complete block in the development of $\gamma\delta$ T cells, NK and NKT cells. By comparison with mouse strains deficient in individual cytokines or cytokine receptors, the cytokines responsible for certain aspects of the γ_c/Jak3 deficiency phenotype have been determined. For example IL-7 is absolutely required for $\gamma\delta$ T cell differentiation, while the absence of IL-15 signaling appears to be solely responsible for the lack of NK and NKT cells [191-194]. The role of individual γ_c cytokines in $\alpha\beta$ T cell differentiation is less clear. While $\alpha\beta$ T cells are reduced in

number in Jak3- and γ_c-deficient mice , partly due to a block at the TN2 ($CD44^+$ $CD25^+$) to the TN3 ($CD44^-$ $CD25^+$) transition, at least some T cells progress through all stages of thymocyte development and become mature $CD4^+$ and $CD8^+$ T lymphocytes [43, 179, 180, 189]. This phenotype is in some respects consistent with that of mice deficient in IL-7 alone, which also have greatly reduced thymocyte numbers suggesting that IL-7 plays a non-redundant role in early thymocyte expansion [185, 186].

One well-known function of IL-7 is the upregulation of the anti-apoptotic factor Bcl-2 [17, 187]. Given the reduction in thymic cellularity that results from the absence of IL-7 signaling, several studies have investigated whether this aspect of the thymic defect in γ_c-deficient mice may be rescued by the enforced expression of Bcl-2. To do this, IL-7-deficient as well as γ_c-deficient mice have been crossed to Bcl-2 transgenic mice that express Bcl-2 under the control of different promoters. However, these investigations have produced conflicting results; whereas some groups claimed that Bcl-2 expression was sufficient to rescue the developmental defect in both IL-7 and γ_c-deficient mice [18, 248, 249], other groups claimed that Bcl-2 expression merely resulted in a limited increase of thymocytes numbers, and did not correct the block observed in the development of thymocyte progenitors in these mice [17, 250]. In an attempt to clarify these findings, I investigated the development of thymocytes in Jak3-deficient mice that expressed Bcl-2 under the Eμ promoter. Consistent with Rodewald et al [250], I observed that even though there was a slight increase in thymocyte numbers in Bcl-2 expressing Jak3-deficient mice, progression from the TN2 to the TN3 stages of development was not rescued by the enforced expression of Bcl-2. Therefore, it appears

that Bcl-2 is necessary for the survival of thymocytes, and its expression leads to an augmentation of thymocyte numbers in Jak3/γ_c-deficient mice. However, since Bcl-2 expression alone is not sufficient to rescue the developmental block between the TN2 and TN3 stages, either additional IL-7-mediated effects, or functions mediated by other γ_c-cytokines are required for the proper development of progenitor cells in mice.

Since it has been demonstrated that Bcl-2 expressed under the H-2K promoter caused the generation of increased thymocyte numbers compared with Bcl-2 expressed under the Eμ promoter, it is possible that the failure to rescue the block in development may have been due to the inability of the Eμ promoter to drive expression of Bcl-2 in early progenitors. However, two independent studies demonstrated that, under the Eμ promoter, Bcl-2 was expressed in all TN stages of development and that its expression led to the *in vitro* survival of these cell populations in the absence of cytokines [250, 253]. Furthermore, analysis of Bcl-2 expression in the absence of IL-7 revealed that Bcl-2 expression at the TN1 ($CD44^+$ $CD25^-$) stage was independent of IL-7 [187]. Because the studies conducted using the H-2K promoter did not include an analysis of the TN stages of development, we do not know whether the higher number of thymocytes observed in this system was simply due to a greater accumulation of thymocytes or an actual rescue in the developmental block. Therefore, while it remains possible that the block in thymocyte development can be overcome if Bcl-2 is expressed by even earlier progenitors or at a higher concentration than that achieved by the Eμ-mediated expression in our model system, I would argue that the level of IL-7-induced expression of Bcl-2 is more consistent with our model, and therefore either that IL-7 mediates

functions other than regulating Bcl-2, or that other γ_c cytokines are involved in regulating the TN2 to TN3 checkpoint.

As mentioned above, some $\alpha\beta$ thymocytes are able to undergo every step of development and mature into $CD4^+$ and $CD8^+$ T cells in Jak3- and γ_c-deficient mice [179-181], such that total numbers of mature T cells appear to be normal. However, the majority of these peripheral T cells are $CD4^+$ and appear to have an activated or memory-like phenotype, characterized by the upregulation of CD44 and the downregulation of CD62-L [179]. Thus, normal T cell homeostasis is highly dysregulated in these mice in terms of both the CD4 to CD8 ratio, as well as the naïve to memory T cell ratio. In the absence of γ_c cytokine signals, naïve $CD4^+$ and $CD8^+$, as well as memory-like $CD8^+$ T cells appear to be virtually absent from the periphery. This is perhaps not unexpected as several reports have demonstrated that IL-7 is necessary, not only for the survival but also for the homeostatic proliferation of naïve T cells [57, 73-75, 212], while both IL-15 and IL-7 are essential for the survival and homeostatic proliferation of $CD8^+$ memory T cells [74, 197, 199, 200]. In contrast, both the survival and homeostatic proliferation of memory $CD4^+$ T cells have been shown to be independent of γ_c cytokines [74, 75]. However, recent reports suggest that the survival of memory $CD4^+$ T cells is somewhat dependent on IL-7-mediated signals [201, 202].

The expansion and activation of $CD4^+$ T cells observed in Jak3-deficient mice could in theory be a result of developmental defects, TCR-dependent activation defects, or some other TCR-independent T cell intrinsic defect. Several previous reports suggest that negative selection during thymocyte development takes place normally in mice

deficient in γ_c-mediated signals [11, 16]. The restoration of Jak3 expression in $Jak3^{-/-}$ thymocytes by introducing a Jak3 transgene under the control of the Lck proximal promoter further discounts a developmental defect as the underlying cause of the disruption of normal peripheral homeostasis [205]. These mice, which express Jak3 only in the thymus, generate normal numbers of thymocytes, but lose Jak3 expression in the peripheral T cells; despite this, the peripheral T cell compartment recapitulates that of non-transgenic $Jak3^{-/-}$ mice and is characterized by the expansion of mature memory-like $CD4^+$ T cells.

These findings suggest that expansion of $CD4^+$ T cells most likely takes place after export from the thymus, but there remains the problem of whether this peripheral $CD4^+$ T cell activation and expansion is dependent on TCR specificity, or represents a cell intrinsic defect. Crossing Jak3- or γ_c-deficient mice to MHC class I- or class II-specific TCR transgenic mice resulted in a significant reduction in peripheral T cell numbers. TCR^+ T cells in these mouse models appeared to have a naïve phenotype, suggesting that TCR-specific signals were required for the activation and expansion of mature T cells in $Jak3^{-/-}$ and $\gamma_c^{-/-}$ mice. I therefore hypothesized that if $CD4^+$ T cell expansion was TCR-specific, only specific T cell clones would populate the periphery of $Jak3^{-/-}$ mice. I investigated this theory using CDR3 spectratype analysis to examine the $CD4^+$ TCR repertoire in the periphery and thymus of Jak3-deficient mice. I observed that the TCR repertoire of $CD4^+$ peripheral T cells in $Jak3^{-/-}$ mice was significantly skewed compared to that of $Jak3^{+/-}$ control $CD4^+$ T cells. This skewing was not observed in $Jak3^{-/-}$ thymocytes, suggesting that the preferential expansion of certain $CD4^+$ T cell clones

took place in the periphery of Jak3-deficient mice and that the activation and expansion of mature T cells in these mice is dependent on the specificity of the TCR.

T cell homeostasis dysregulation in Jak3-deficient mice

From the results described above, I propose the following model to explain the dysregulation in T cell homeostasis characteristic of mice deficient in Jak3/γ_c-mediated signals. During development, a very low number of precursor DN thymocytes begin to rearrange the TCR β chain, committing to the αβ T cell lineage. Small numbers of these cells proceed through every stage of thymic development and are normally selected into mature SP $CD4^+$ and $CD8^+$ T cells. The reduced numbers of $CD8^+$ T cells in Jak3-deficient mice maybe initiated at the $CD8^+$ SP thymocyte stage as these cells have been shown to be more dependent on Bcl-2 and Bcl-xL (induced by IL-7 and IL-15) for their survival [190] (Fig.22).

Having completed development and entered the periphery where they circulate to secondary lymphoid organs, mature naïve $CD4^+$ and $CD8^+$ T cells normally require signals mediated through the IL-7R in order to survive. Therefore, most naïve $Jak3^{-/-}$ $CD4^+$ and $CD8^+$ T cells, lacking the ability to receive IL-7 signals, will die shortly after entering the periphery. However, certain $Jak3^{-/-}$ T cell clones may be rescued from this fate if they receive stronger TCR signals, through recognition of self-peptides or environmental Ags found in the gut flora that might compensate for the lack of IL-7 signals. The Ag-driven activation and expansion of these cells would effectively rescue them from their dependence on IL-7 survival. Ag-driven activation of $CD4^+$ T cells in

Jak3$^{-/-}$ mice could be exacerbated by the lack of CD25^{+}CD4^{+} regulatory T cells. A few studies have suggested that the T cell expansion and autoimmunity observed in IL-2-deficient mice can be reverted by the adoptive transfer of CD25^{+} CD4^{+} regulatory T cells. Development of these regulatory T cells is dependent on IL-2, and thus they are absent in IL-2-deficient mice [132, 133]. Whether or not Jak3-deficient mice have CD25^{+} CD4^{+} regulatory T cells has not been elucidated, but recent data from our lab suggests that Jak3$^{-/-}$ mice are deficient in these cells. Additionally, the reduced numbers of thymocytes generated in Jak3$^{-/-}$ mice results in a significantly decreased rate of thymic export into the periphery in Jak3$^{-/-}$ compared to WT mice. This means that emerging Jak3$^{-/-}$ lymphocytes enter an essentially empty immune compartment. The presence of this "space" may trigger homeostatic proliferation of the CD4^{+}CD44^{hi} memory-like T cells, bringing the total numbers of T cells to normal levels to fill the available space, comparable to the expansion seen when small numbers of normal T cells transferred into a lymphopenic environment. Cells that have undergone homeostatic proliferation have been shown to survive for longer periods of time in the absence of γ_c cytokine signals. Furthermore, activated or memory-like CD4^{+} T cells in these mice may not be able to undergo AICD under certain circumstances, due to a defect in IL-2-mediated FasL expression. Even if memory CD4^{+} T cells cannot survive for long periods of time, their death may be masked by their continuous proliferation due to the reasons discussed above. In fact, Jak3$^{-/-}$ mature T cells exhibit higher levels of proliferation and higher levels of apoptosis *in vivo* [273]. Surprisingly, despite the similarities observed during thymic development between Jak3- and IL-7-deficient mice, IL-7-deficient mice do not present the peripheral

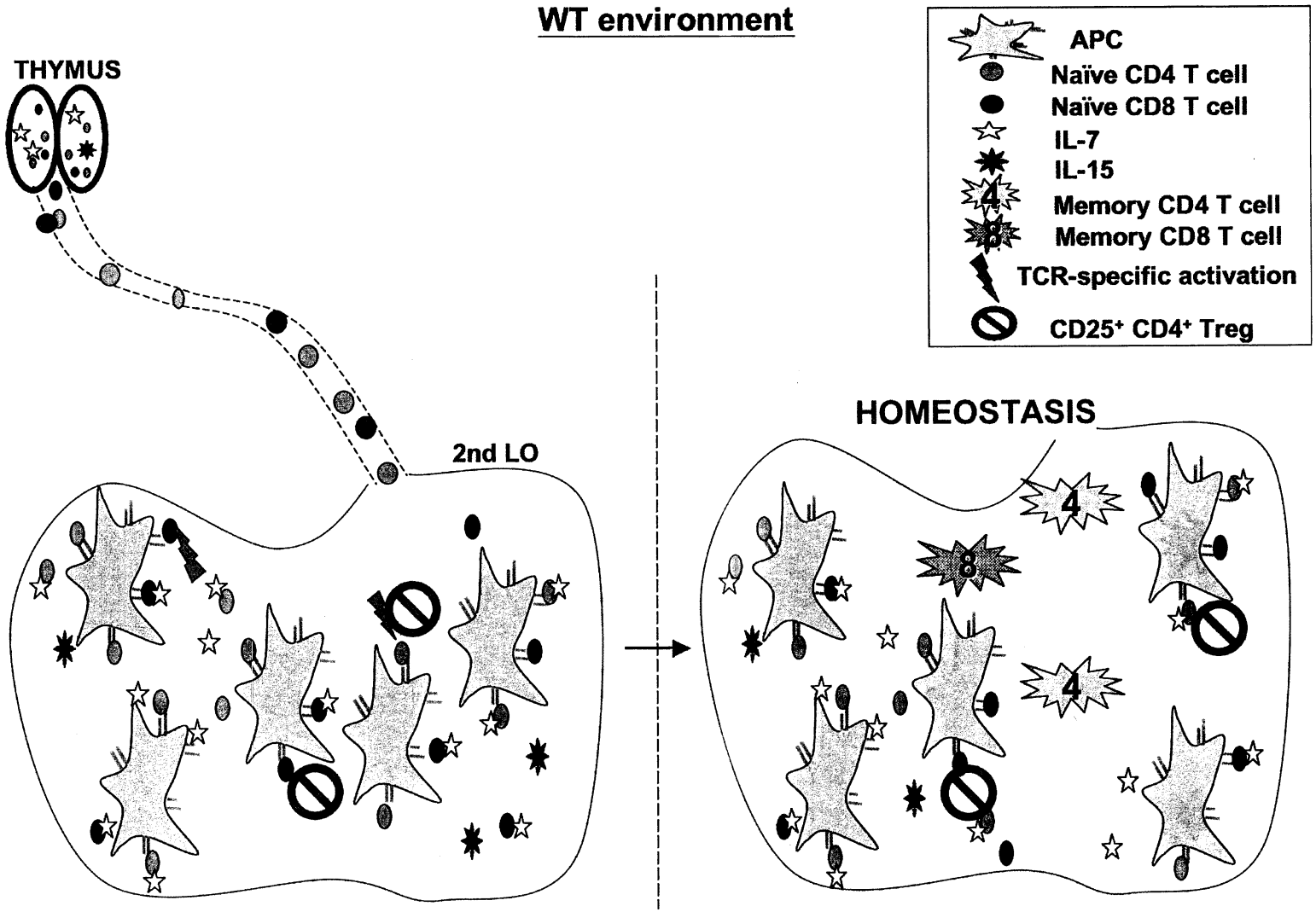
$CD4^+$ T cell expansion characteristic of $Jak3^{-/-}$ mice. In theory, the periphery of these mice should provide the necessary space for T cells to undergo homeostatic proliferation, therefore, factors other than lymphopenia are required for the activation and expansion of $CD4^+$ T cells in Jak3-deficient mice. Since the development of CD4+ CD25+ regulatory T cells appears to be dependent on IL-2 alone, it is possible that IL-7-deficient mice still produce enough levels of these cells, resulting in the regulation of peripheral T cell activation. Additionally, IL-7-deficient T cells should not be defective in their ability to upregulate FasL, which may also contribute to the regulation of T cell activation. Finally, the environment resulting from the lack of Jak3 may be more conducive to T cell activation than the IL-7-deficient environment. For example, $Jak3^{-/-}$ mice may produce elevated levels of type I interferons which could lead to the activation of APCs and thus to the activation and expansion of T cells. In support of this hypothesis I observed that the LCMV titers during the early phase of the infection were lower in $Jak3^{-/-}$ mice compared to $Jak3^{+/-}$ control mice, this could be a result of higher levels of type I interferons. Furthermore, recent observations by Joonsoo Kang's group suggest that signals mediated through the STAT1 pathway may be dysregulated in the absence of STAT5 signals.

Memory $CD8^+$ T cells are dependent on signals mediated through the IL-15 and IL-7 receptors to survive and homeostatically proliferate. Consequently, these cells would also fail to survive in Jak3-deficient mice. In contrast, $CD4^+$ memory T cells are capable of undergoing homeostatic proliferation in the absence of γ_c cytokines and only their survival appears to be partly dependent on IL-7.

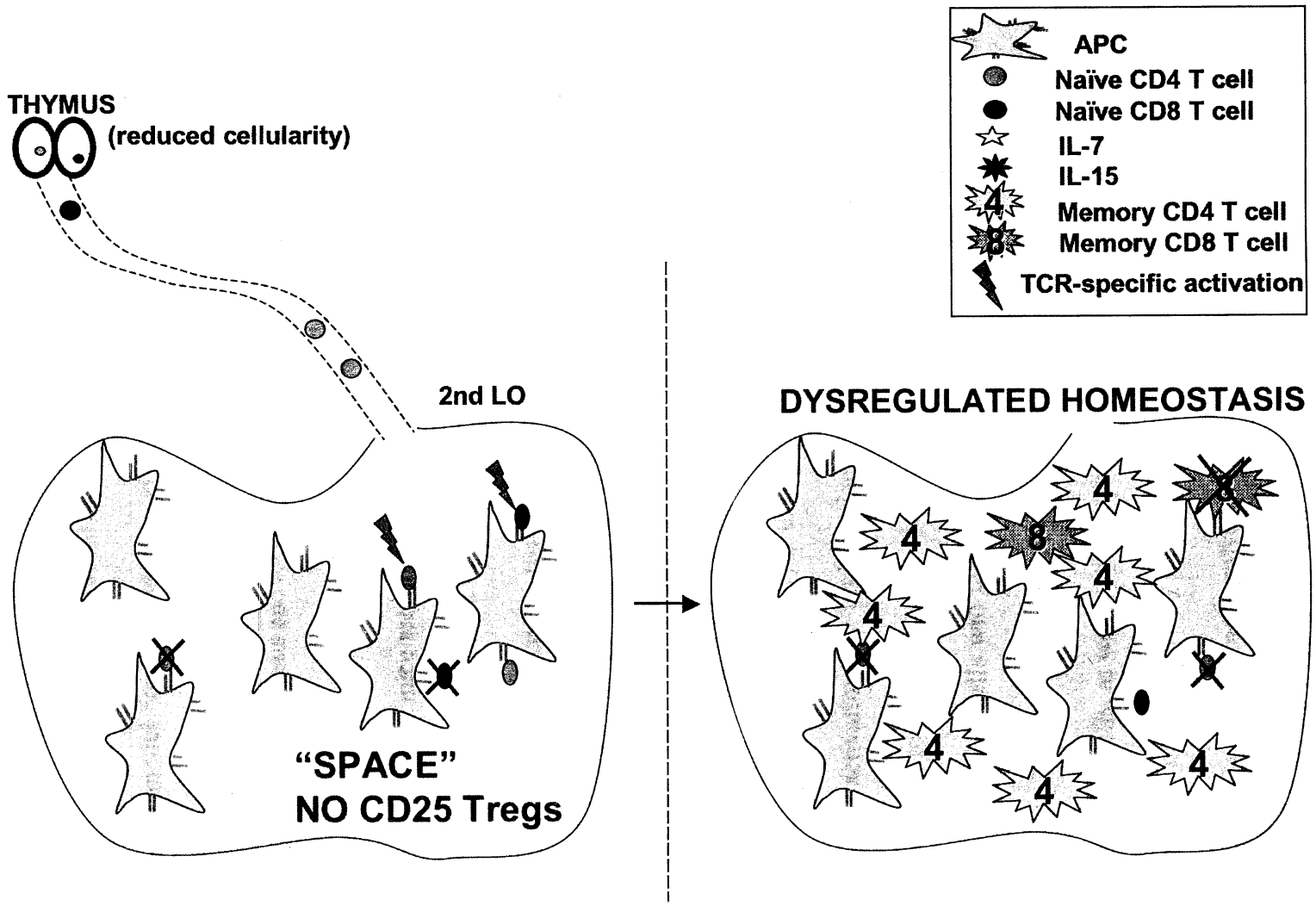
Consistent with this model, the periphery of Jak3-deficient mice is characterized by an almost complete absence of $CD8^+$ T cells and practically normal numbers of $CD4^+$ T cells, most of which present an activated or memory-like phenotype. In contrast, the periphery of $Jak3^{+/-}$ mice is characterized by the presence of normal numbers of $CD4^+$ and $CD8^+$ naïve T cells, a constant ratio of CD4 to CD8 T cells (~2:1), and a constant ratio of naïve (~75-80%) to memory T cells (~15-20%).

Fig.22 Dysregulated T cell homeostasis in Jak3$^{-/-}$ mice

A model for the dysregulation in T cell homeostasis observed in Jak3-deficient mice compared to WT mice is depicted in the following pages and was discussed in the previous pages. The first page of the model describes T cell homeostasis in a WT mice. The second page describes my model for the dysregulated homeostasis characteristic of Jak3$^{-/-}$ mice.


WT environment
APC
Naïve CD4 T cell
Naïve CD8 T cell
IL-7
IL-15
Memory CD4 T cell
Memory CD8 T cell
TCR-specific activation
CD25+ CD4+ Treg
THYMUS
2nd LO
HOMEOSTASIS
4
8
4

Jak3-deficient environment

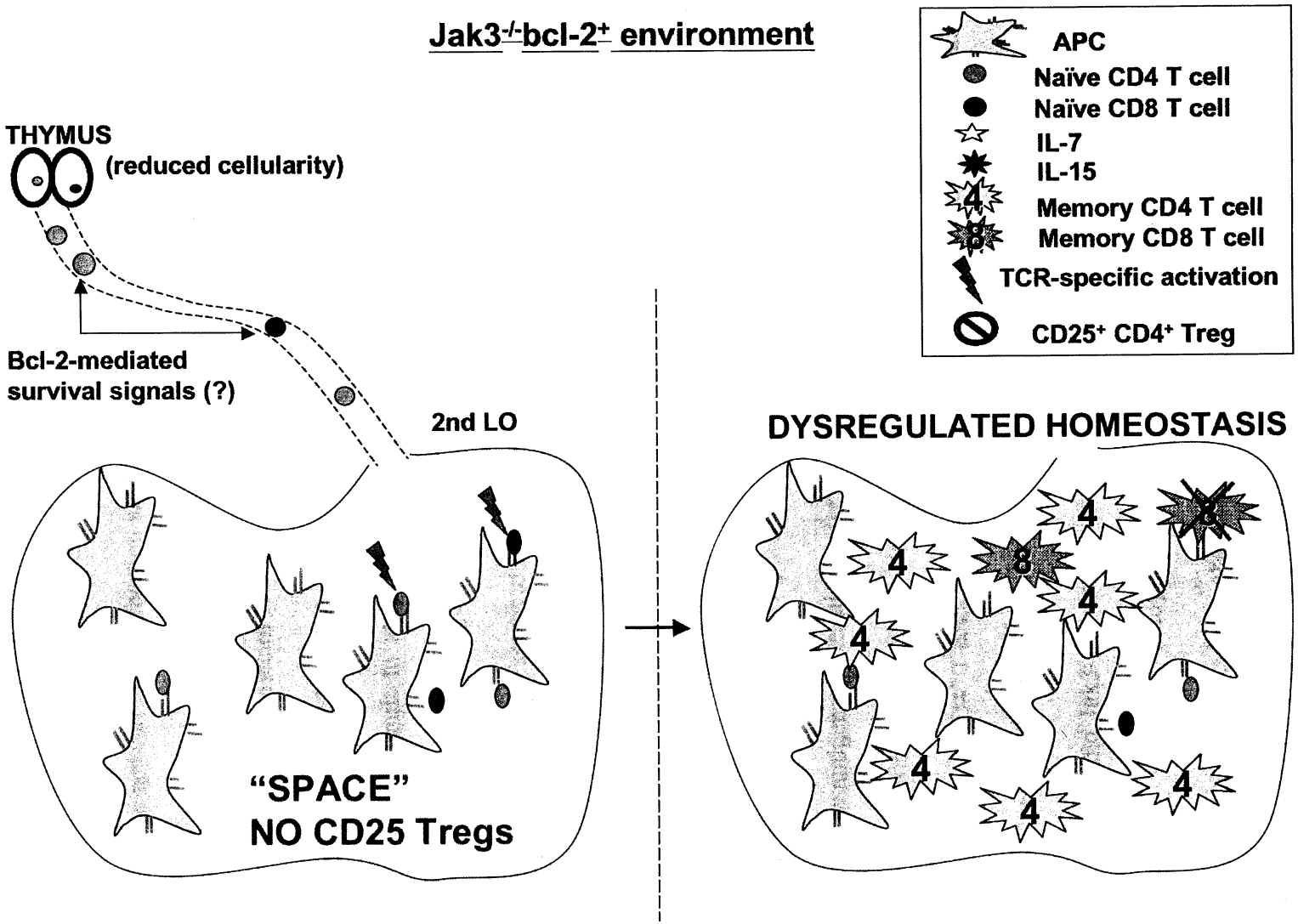


T cell homeostasis dysregulation in Bcl-2 expressing Jak3-deficient mice

From the results obtained upon analysis of Jak3$^{-/-}$bcl-2^{+} mice I propose the following model for the homeostasis defect observed in these mice. The enforced expression of Bcl-2 does not rescue the developmental defect in the absence of Jak3/γ_c–mediated signals and total thymocyte numbers are greatly reduced in Jak3$^{-/-}$bcl-2^{+} mice. Consequently, the total numbers of CD4 and CD8 mature T cells circulating in the periphery of these mice are still very reduced compared to WT mice. Bcl-2 may assist in the survival of naïve T cells in this system, but the levels of Bcl-2 may not be sufficient or additional actors may be required for the survival of naïve T cells. Additionally, engagement of TCRs from certain T cell clones with self- or foreign-peptide-MHC complexes will result in their activation, as in WT mice which present a significant percentage of memory-like CD4^{+} and CD8^{+} T cells under normal, pathogen-free circumstances. Additionally, if these mice are deficient in CD25^{+} CD4^{+} regulatory T cells, the activation and proliferation of mature T cells will be highly dysregulated. Finally, due to the reduced numbers of mature peripheral T cells in Jak3$^{-/-}$bcl-2^{+} mice, CD4^{+} CD44^{hi} T cells will undergo homeostatic proliferation, thus becoming the most prominent T cell population in the periphery of these mice. The numbers of naïve CD4^{+} and CD8^{+} T cells, as well as the numbers of memory CD8^{+} T cells higher in Jak3$^{-/-}$bcl-2^{+} than Jak3$^{-/-}$ mice. This may be a consequence of the enforced expression of Bcl-2, since IL-7 signals that promote naïve T cell survival would normally upregulate Bcl-2 in mature naïve T cells. Nevertheless, additional mechanisms other than Bcl-2 expression are probably required for the survival of mature T cells (Fig.23).

Fig.23 Dysregulated T cell homeostasis in Jak3$^{-/-}$bcl-2^{+} mice

This figure depicts the model described in the previous page, of dysregulated T cell homeostasis in the periphery of Jak3-deficient mice that express Bcl-2 under the Eμ promoter.


Jak3-/-bcl-2+ environment
APC
Naïve CD4 T cell
Naïve CD8 T cell
IL-7
IL-15
Memory CD4 T cell
Memory CD8 T cell
TCR-specific activation
CD25+ CD4+ Treg
THYMUS
(reduced cellularity)
Bcl-2-mediated
survival signals (?)
2nd LO
"SPACE"
NO CD25 Tregs
DYSREGULATED HOMEOSTASIS

Cytokines are key players in the development of adaptive immune responses, controlling the type, the intensity and the kinetics of a response by specifically inducing or inhibiting the activation, proliferation and differentiation of immune cells [112, 113, 274, 275]. Cytokines of the γ_c family play important roles in all aspects of T cell activation, proliferation, effector function and memory production. However, due to the severe developmental defects that result from the absence of γ_c-mediated signals, it has been extremely difficult to study their role in T cell activation and effector cell differentiation. A few years ago, Di Santo's group observed that $\gamma_c^{-/-}$ TCR transgenic $CD4^+$ T cells were able to respond to a specific Ag stimulation and proliferate to the same levels and with the same kinetics as WT control cells. They also observed that transferring already primed $\gamma_c^{-/-}$ TCR^+ $CD4^+$ T cells into alymphoid mice resulted in their ability to respond to antigenic re-challenge and to survive for at least 5 weeks. Therefore they concluded that γ_c-mediated signals were required for the survival of naïve $CD4^+$ T cells but not for antigen proliferation or for the maintenance and restimulation of memory $CD4^+$ T cells [206]. However, recent results suggest that both the production and survival of memory $CD4^+$ T cells may, at least in part, be dependent on signals mediated through the γ_c chain [201, 202].

Since γ_c- and Jak3-deficient mice have extremely reduced numbers of mature $CD8^+$ T cells. Therefore, studying the role of γc cytokine signals in $CD8^+$ T cell activation has been particularly challenging. Most previous analyses of γ_c cytokine involvement in $CD8^+$ T cell activation has been conducted using mouse models deficient in only one or two cytokines from the family. However, these results remain

controversial. While it is agreed that IL-2 or IL-15 is essential for mounting a $CD8^+$ T cell response, the degree to which their absence affects the full development of effector function remains contentious. Whereas some reports suggest that neither cytokine is required for the full $CD8^+$ T cell differentiation, others have argued that IL-2 and IL-15 determine the intensity of the response and are necessary for full differentiation of effector CTLs. Of all the γ_c cytokines, IL-2 and IL-15 are the most likely candidates to act as mediators of T cell activation, but IL-4, IL-7 and IL-21 may play an accessory role, and may even compensate, in the absence of the former cytokines. The role played by other γ_c cytokines, such as IL-9, in T cell activation has not been fully investigated. In order to circumvent possible cytokine redundancies and further elucidate the role played by all γ_c cytokines during T cell activation, I investigated the ability of Jak3-deficient mice to mount a specific $CD8^+$ T cell response to viral infection.

To this end I infected Jak3-deficient mice with LCMV, a broadly studied model of infection that results in a massive and well characterized $CD8^+$ T cell response. Interestingly, only 40% of the $Jak3^{-/-}$ mice challenged with LCMV were able to mount a significant $CD8^+$ T cell response. Furthermore, $Jak3^{-/-}$ $CD8^+$ T cells that did initiate a response failed to expand and their effector function was greatly impaired.

There are several possible explanations for the inability of a major proportion of $Jak3^{-/-}$ mice to mount a visible $CD8^+$ T cell response upon LCMV infection, but the most likely explanation is the reduced availability of LCMV specific T cell clones. As previously discussed, $Jak3^{-/-}$ mice have highly reduced numbers of $CD8^+$ T cells. Further, these cells most likely turn over extremely rapidly due to their inability to survive in the

absence of IL-7 and IL-15 signals. Therefore, it is possible that the ability of Jak3-deficient mice to respond to LCMV depends largely on the frequency (or total absence) of LCMV-specific T cell clones available in the limited $CD8^+$ TCR repertoire present at the time of infection. Nevertheless, it is important to note that some $Jak3^{-/-}$ mice were able to make a measurable response to LCMV, indicating that even though these responses were highly impaired and insufficient to clear the virus, γ_c signals are not absolutely required for the initial activation of $CD8^+$ T cells. These results are in agreement with recent reports suggesting a role for IL-2 and IL-15 during the expansion phase of $CD8^+$ T cell mediated viral responses but not during the initial activation [211, 212]. The defects in Jak3-deficient mice are much more severe than those obtained with IL-2 and IL-15-deficient mice, most likely due to the absence of IL-7, IL-4 and IL-21. Specially IL-21 has recently been shown to improve $CD8^+$ T cell responses by enhancing their ability to lyse target cells and produce IFN-γ [272, 276].

Interestingly, the impaired function of LCMV-specific $Jak3^{-/-}$ T cells is reminiscent of the phenotype of activated $CD8^+$ T cells during a chronic infection. In two different models of chronic viral infections with LCMV, perforin knock-out mice and WT mice infected with LCMV clone 13, $CD8^+$ T cells underwent a hierarchical loss of functionality similar to what I observe in LCMV-infected Jak3-deficient mice [270]. Activated $Jak3^{-/-}$ $CD8^+$ T cells make IFN-γ but are incapable of making TNF-α or granzyme B. This prevents them from properly lysing target cells. Similarly, while IL-2 and TNF-α production, as well as cell-mediated cytotoxicity were highly impaired during chronic infection, IFN-γ production was more resistant.

The inability of $CD8^+$ T cells in $Jak3^{-/-}$ mice to mount a successful $CD8^+$ T cell response is unlikely to be solely the direct result of the complete absence of Jak3/γ_c-mediated signals in $CD8^+$ T cells themselves. Alternatively, or additionally, factors other than the $CD8^+$ T cell intrinsic defects could influence the ability of Jak3-deficient mice to generate a robust CTL response. Firstly, the initiation of an innate response against the virus may have been impaired in $Jak3^{-/-}$ mice. In particular, the lack of mature NK cells has the ability to influence the generation of CTL responses at a number of levels. During certain viral infections, NK cells may limit the viral load by producing cytokines, such as IFN-γ and TNF that inhibit viral replication and by direct lysis of infected cells [274, 277]. Lysis of infected cells may also contribute to the uptake of Ag. by DCs. Additionally, they produce chemokines that result in the recruitment of DCs and other immune cells, including activated $CD8^+$ T cells, to the site of infection [274, 277] However, recent reports have shown that IL-15- and IL-15R-deficient mice, which also lack NK cells, can make potent primary responses to LCMV, suggesting that the absence of NK cells alone should not preclude the induction of an immune response. Nevertheless, this possibility could be addressed in future experiments by adoptively transferring WT $CD8^+$ T cells into Jak3-deficient mice and investigating their ability to become activated and clear a viral infection, such as LCMV.

In addition, the loss of $CD8^+$ DCs in adult $Jak3^{-/-}$ mice (Wallace ME, unpublished data) could influence the ability of these mice to develop functional CTLs. $CD8^+$ DCs are major producers of IL-12, a cytokine known to promote type I immune responses and induce IFN-γ production [278]. However, recent reports suggest that IL-12-deficient

DCs can successfully prime $CD8^+$ T cell effector function, as measured by cytotoxicity and IFN-γ production *in vivo* [279]. Therefore, the lack of $CD8^+$ DCs in Jak3-deficient mice may be of limited importance. One way to directly test this possibility would be by injecting WT $CD8^+$ DCs into $Jak3^{-/-}$ mice prior to a viral infection.

The LCMV model, though physiologically relevant, is complicated by a variety of external factor in the Jak3-deficient environment (as explained above) that could be influencing the generation of the $CD8^+$ T cell response, making it difficult to assess the intrinsic role of Jak3-mediated cytokine signals in $CD8^+$ T cell activation and differentiation. To circumvent these experimental limitations, I generated OT-1^+bcl-2^+ mice that produce a significant population of naïve $CD8^+$ T cells of known specificity. This allowed me to isolate equivalent numbers of $CD8^+$ Vα-2^+ T cells from either $Jak3^{-/-}$ or control mice, and either stimulate them *in vitro* or transfer them into congenic WT mice and follow their activation in a more controlled environment. These types of studies had not previously been possible because of the difficulties associated with isolating sufficient numbers of $CD8^+$ T cells from $Jak3^{-/-}$ or TCR transgenic $Jak3^{-/-}$ mice. I observed that, both *in vitro* and *in vivo,* $CD8^+$ T cells from Jak3-deficient mice were able to become activated and initiate a specific immune response, however this response was greatly diminished, in terms of T cell expansion and differentiation into functional cytokine-producing effector cells, compared to WT cells. Comparable to the results obtained with the LCMV model, in response to a specific viral challenge, Jak3-deficient $CD8^+$ T cells can be activated, proliferate and differentiate into IFN-γ producing effector cells. However, their proliferation is impaired compared to WT control $CD8^+$ T cells,

and their survival rate is greatly diminished, most likely due to the lack of IL-2, IL-15 and IL-7 signals.

SIGNIFICANCE

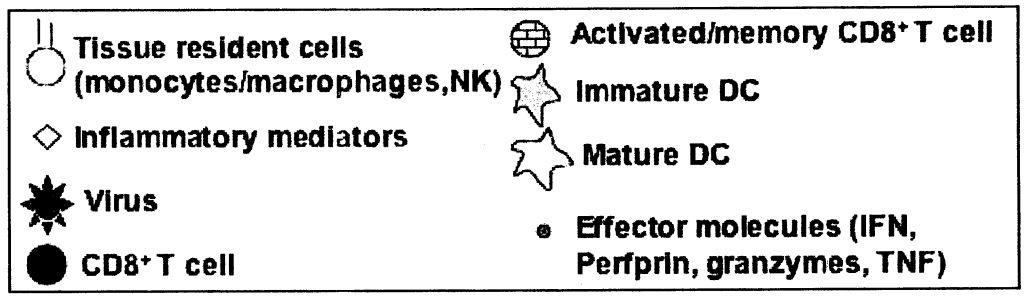
During a viral infection, the innate arm of the immune system is activated, and different cell types, such as macrophages and NK cells release inflammatory mediators, including chemokines that attract immature DCs to the site of pathogen entry. DCs pick up Ag, mature and travel to the secondary lymphoid organs where they activate naïve T cells that express TCRs specific for the infecting pathogen, thus initiating an adaptive immune response. Upon activation, T cells downregulate and upregulate different receptors that guide them away from the lymphoid organs into the site of infection. They also differentiate into effector cells capable of lysing infected cells and producing essential cytokines that further mediate their expansion and ability to clear the infection. Immediately after the infection is cleared, most of the activated cells undergo activation induced cell death, which is mediated by different signaling pathways, while also producing a small population of memory cells (Fig.23). The following model describes the role of γc cytokine signals in $CD8^+$ T cell activation and function as predicted from the studies of $CD8^+$ T cell activation and function described in this thesis.

In the absence of Jak3 or γ_c, mature $CD8^+$ T cells are unable to survive in the periphery for any length of time, due to their requirement for IL7 to survive as naïve cells and IL-7/IL-15 to survive as memory cells. Therefore, the small number of $CD8^+$ T cells present in the periphery at any point in time, most likely represent very recent thymic

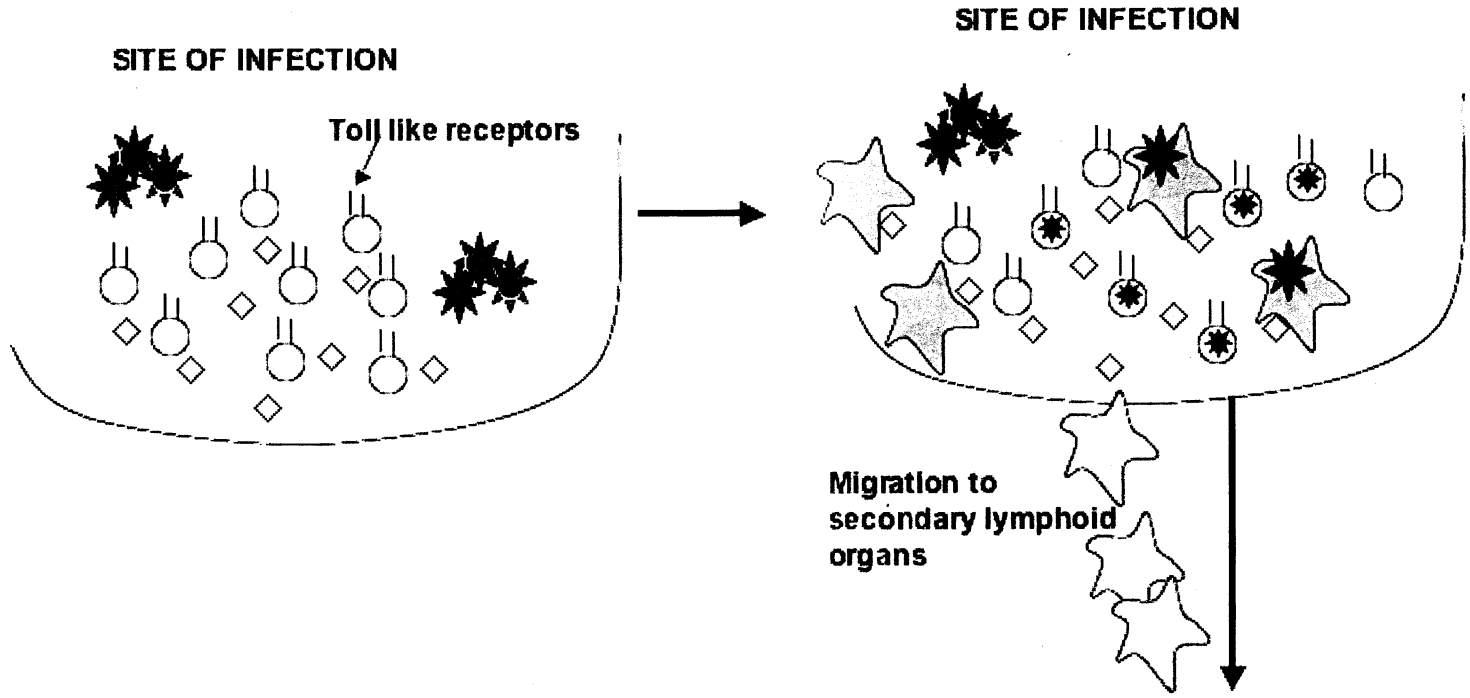
emigrants or maybe cells that have a higher affinity for self-MHC-self-peptide complexes. On infection with a virus, deficiencies in the innate immune system of $Jak3^{-/-}$ mice may reduce the ability to properly activate the adaptive arm of the immune system. Notwithstanding these external factors, it is clear from our data that despite the very low numbers of $CD8^+$ T cells present, at least some activation of $CD8^+$ T cells is possible in the absence of γ_c signals. Given the very limited repertoire of $CD8^+$ T cells available in certain cases, $Jak3^{-/-}$ mice may be completely devoid of virus-specific $CD8^+$ T cells. This absence of virus-specific T cell clones results in a lack of $CD8^+$ T cell expansion and differentiation following the viral infection. If LCMV clones are present their activation is followed by limited expansion and partial differentiation into effector $CD8^+$ T cells. However, due to all of these factors, the reduced numbers of virus-specific T cell clones, their inability to differentiate into functional effector cells, and their inability to lyse cells, $Jak3^{-/-}$ mice are incapable of controlling the infection. This results in a state of chronic viral infection or death of the mice, depending on the infecting pathogen (Fig. 24).

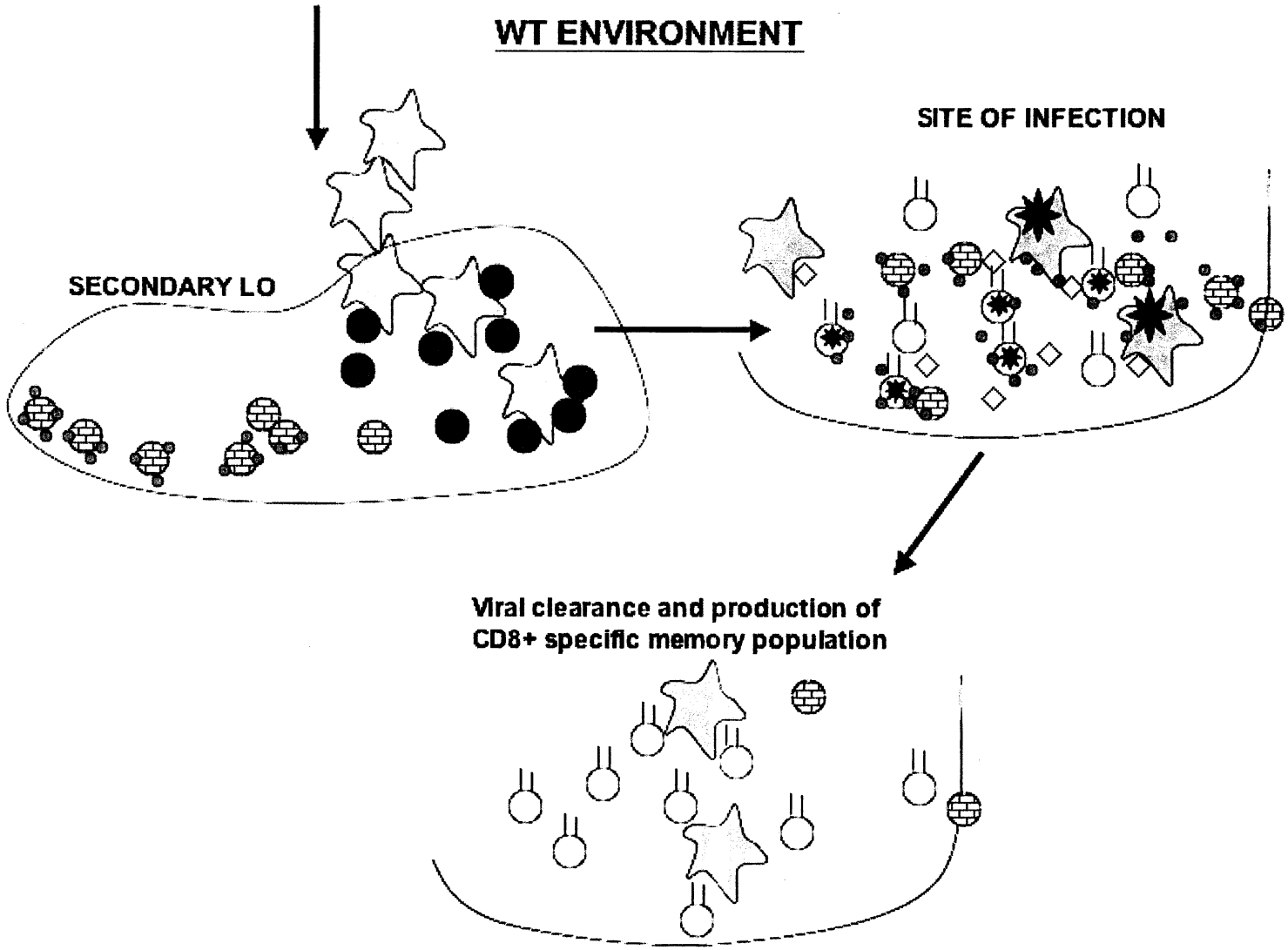
Fig.24 Antiviral responses by $CD8^+$ T cells in the absence of Jak3

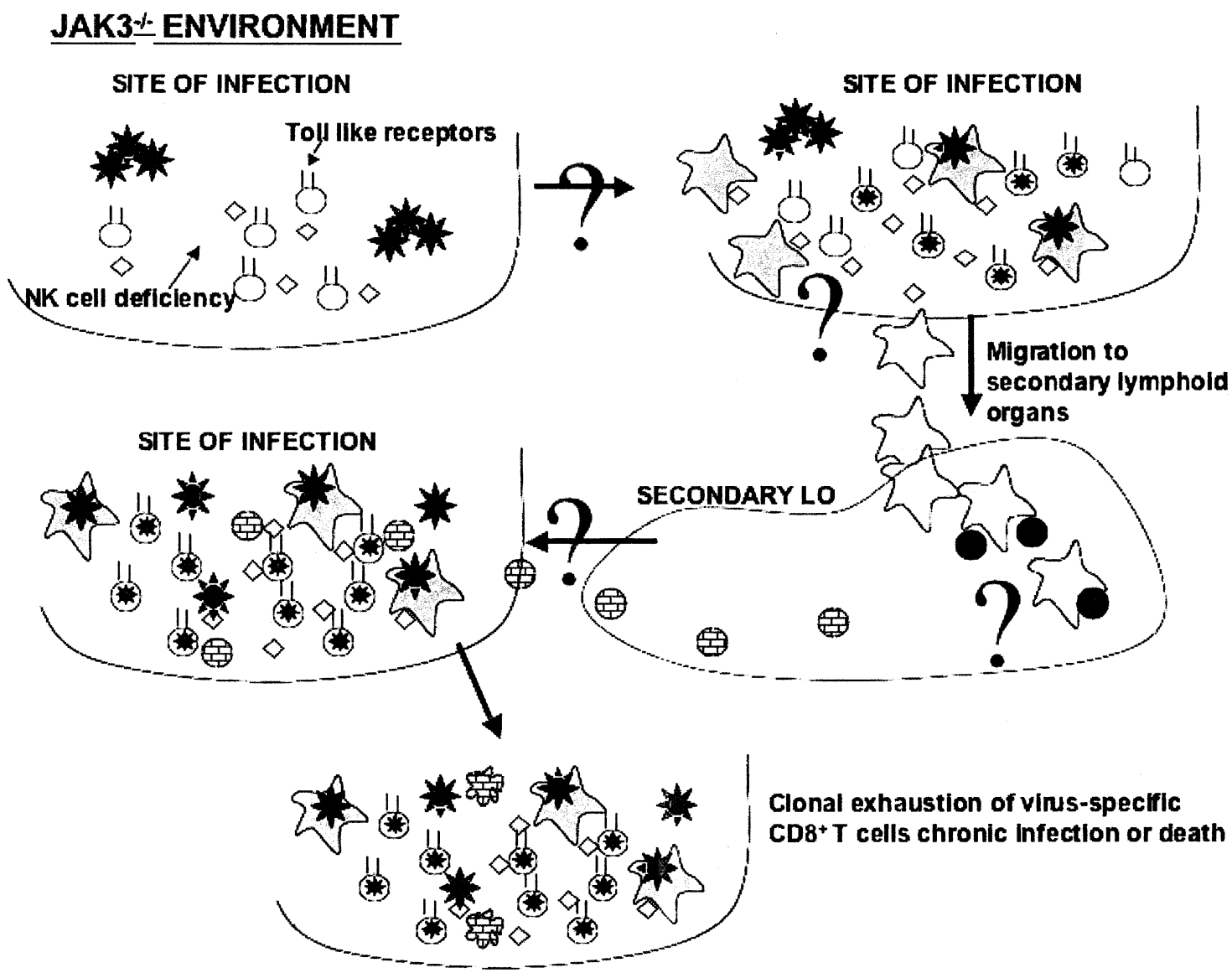
The following pages describe the working model the development of a CD8-mediated immune response in the absence of γ_c-dependent cytokines. The first two pages depict a response mediated by $CD8^+$ T cells in a WT mouse. The third page depicts the immune response mediated by $CD8^+$ T cells in a Jak3-deficient mouse, against a viral infection.



WT ENVIRONMENT




WT ENVIRONMENT
SECONDARY LO
SITE OF INFECTION
Viral clearance and production of
CD8+ specific memory population


JAK3-/- ENVIRONMENT
SITE OF INFECTION
Toll like receptors
NK cell deficiency
SITE OF INFECTION
Migration to secondary lymphoid organs
SECONDARY LO
SITE OF INFECTION
Clonal exhaustion of virus-specific CD8+ T cells chronic infection or death

CHAPTER VII

LITERATURE CITED

1. Rolink, A.G., et al., *Selection events operating at various stages in B cell development.* Curr Opin Immunol, 2001. **13**(2): p. 202-7.

2. Davis, M.M. and P.J. Bjorkman, *T-cell antigen receptor genes and T-cell recognition.* Nature, 1988. **334**(6181): p. 395-402.

3. Robey, E. and B.J. Fowlkes, *Selective events in T cell development.* Annu Rev Immunol, 1994. **12**: p. 675-705.

4. Raulet, D.H., et al., *Developmental regulation of T-cell receptor gene expression.* Nature, 1985. **314**(6006): p. 103-7.

5. Shortman, K. and L. Wu, *Early T lymphocyte progenitors.* Annu Rev Immunol, 1996. **14**: p. 29-47.

6. Lo, D., Y. Ron, and J. Sprent, *Induction of MHC-restricted specificity and tolerance in the thymus.* Immunol Res, 1986. **5**(3): p. 221-32.

7. Goldrath, A.W. and M.J. Bevan, *Selecting and maintaining a diverse T-cell repertoire.* Nature, 1999. **402**(6759): p. 255-62.

8. Nikolic-Zugic, J., *Phenotypic and functional stages in the intrathymic development of alpha beta T cells.* Immunol Today, 1991. **12**(2): p. 65-70.

9. Boyd, R.L. and P. Hugo, *Towards an integrated view of thymopoiesis.* Immunol Today, 1991. **12**(2): p. 71-9.

10. Fehling, H.J. and H. von Boehmer, *Early alpha beta T cell development in the thymus of normal and genetically altered mice.* Curr Opin Immunol, 1997. **9**(2): p. 263-75.

11. DiSanto, J.P., et al., *Critical role for the common cytokine receptor gamma chain in intrathymic and peripheral T cell selection.* J Exp Med, 1996. **183**(3): p. 1111-8.

12. Germain, R.N., *T-cell development and the CD4-CD8 lineage decision.* Nat Rev Immunol, 2002. **2**(5): p. 309-22.

13. Gratiot-Deans, J., et al., *Bcl-2 expression during T-cell development: early loss and late return occur at specific stages of commitment to differentiation and survival.* Proc Natl Acad Sci U S A, 1994. **91**(22): p. 10685-9.

14. Veis, D.J., et al., *Expression of the Bcl-2 protein in murine and human thymocytes and in peripheral T lymphocytes.* J Immunol, 1993. **151**(5): p. 2546-54.

15. Moore, N.C., et al., *Developmental regulation of bcl-2 expression in the thymus.* Immunology, 1994. **81**(1): p. 115-9.

16. Nakajima, H., M. Noguchi, and W.J. Leonard, *Role of the common cytokine receptor gamma chain (gammac) in thymocyte selection.* Immunol Today, 2000. **21**(2): p. 88-94.

17. Nakajima, H. and W.J. Leonard, *Role of Bcl-2 in alpha beta T cell development in mice deficient in the common cytokine receptor gamma-chain: the requirement for Bcl-2 differs depending on the TCR/MHC affinity.* J Immunol, 1999. **162**(2): p. 782-90.

18. Kondo, M., et al., *Bcl-2 rescues T lymphopoiesis, but not B or NK cell development, in common gamma chain-deficient mice.* Immunity, 1997. **7**(1): p. 155-62.

19. von Boehmer, H., *Positive selection of lymphocytes.* Cell, 1994. **76**(2): p. 219-28.

20. Godfrey, D.I. and A. Zlotnik, *Control points in early T-cell development.* Immunol Today, 1993. **14**(11): p. 547-53.

21. Groettrup, M., et al., *A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor beta chain and a 33 kd glycoprotein.* Cell, 1993. **75**(2): p. 283-94.

22. van Oers, N.S., H. von Boehmer, and A. Weiss, *The pre-T cell receptor (TCR) complex is functionally coupled to the TCR-zeta subunit.* J Exp Med, 1995. **182**(5): p. 1585-90.

23. Borgulya, P., et al., *Exclusion and inclusion of alpha and beta T cell receptor alleles.* Cell, 1992. **69**(3): p. 529-37.

24. Merkenschlager, M., et al., *How many thymocytes audition for selection?* J Exp Med, 1997. **186**(7): p. 1149-58.

25. Mombaerts, P., et al., *Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages.* Nature, 1992. **360**(6401): p. 225-31.

26. Zinkernagel, R.M. and P.C. Doherty, *MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness.* Adv Immunol, 1979. **27**: p. 51-177.

27. Bevan, M.J., *In a radiation chimaera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells.* Nature, 1977. **269**(5627): p. 417-8.

28. Love, P.E. and A.C. Chan, *Regulation of thymocyte development: only the meek survive.* Curr Opin Immunol, 2003. **15**(2): p. 199-203.

29. Amsen, D. and A.M. Kruisbeek, *Thymocyte selection: not by TCR alone.* Immunol Rev, 1998. **165**: p. 209-29.

30. Marrack, P. and J. Kappler, *Positive selection of thymocytes bearing alpha beta T cell receptors.* Curr Opin Immunol, 1997. **9**(2): p. 250-5.

31. Zerrahn, J., W. Held, and D.H. Raulet, *The MHC reactivity of the T cell repertoire prior to positive and negative selection.* Cell, 1997. **88**(5): p. 627-36.

32. Jameson, S.C. and M.J. Bevan, *T cell receptor antagonists and partial agonists.* Immunity, 1995. **2**(1): p. 1-11.

33. Hogquist, K.A., et al., *T cell receptor antagonist peptides induce positive selection.* Cell, 1994. **76**(1): p. 17-27.

34. Margulies, D.H., *Immunology. An affinity for learning.* Nature, 1996. **381**(6583): p. 558-9.

35. Allen, P.M., *Peptides in positive and negative selection: a delicate balance.* Cell, 1994. **76**(4): p. 593-6.

36. Sawada, S., et al., *A lineage-specific transcriptional silencer regulates CD4 gene expression during T lymphocyte development.* Cell, 1994. **77**(6): p. 917-29.

37. Ellmeier, W., et al., *An enhancer that directs lineage-specific expression of CD8 in positively selected thymocytes and mature T cells.* Immunity, 1997. **7**(4): p. 537-47.

38. Ellmeier, W., S. Sawada, and D.R. Littman, *The regulation of CD4 and CD8 coreceptor gene expression during T cell development.* Annu Rev Immunol, 1999. **17**: p. 523-54.

39. Kim, H.K. and G. Siu, *The notch pathway intermediate HES-1 silences CD4 gene expression.* Mol Cell Biol, 1998. **18**(12): p. 7166-75.

40. Kisielow, P., et al., *Positive selection of antigen-specific T cells in thymus by restricting MHC molecules.* Nature, 1988. **335**(6192): p. 730-3.

41. Berg, L.J., et al., *Antigen/MHC-specific T cells are preferentially exported from the thymus in the presence of their MHC ligand.* Cell, 1989. **58**(6): p. 1035-46.

42. Eynon, E.E., et al., *Distinct effects of Jak3 signaling on alphabeta and gammadelta thymocyte development.* J Immunol, 1999. **162**(3): p. 1448-59.

43. DiSanto, J.P., et al., *Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor gamma chain.* Proc Natl Acad Sci U S A, 1995. **92**(2): p. 377-81.

44. Berg, L.J. and J. Kang, *Molecular determinants of TCR expression and selection.* Curr Opin Immunol, 2001. **13**(2): p. 232-41.

45. Fine, J.S. and A.M. Kruisbeek, *The role of LFA-1/ICAM-1 interactions during murine T lymphocyte development.* J Immunol, 1991. **147**(9): p. 2852-9.

46. Anderson, G., et al., *Cellular interactions in thymocyte development.* Annu Rev Immunol, 1996. **14**: p. 73-99.

47. Durum, S.K., et al., *Interleukin 7 receptor control of T cell receptor gamma gene rearrangement: role of receptor-associated chains and locus accessibility.* J Exp Med, 1998. **188**(12): p. 2233-41.

48. Kang, J., M. Coles, and D.H. Raulet, *Defective development of gamma/delta T cells in interleukin 7 receptor-deficient mice is due to impaired expression of T cell receptor gamma genes.* J Exp Med, 1999. **190**(7): p. 973-82.

49. Di Santo, J.P., et al., *The common cytokine receptor gamma chain and the pre-T cell receptor provide independent but critically overlapping signals in early alpha/beta T cell development.* J Exp Med, 1999. **189**(3): p. 563-74.

50. DiSanto, J.P., *Cytokines: shared receptors, distinct functions.* Curr Biol, 1997. **7**(7): p. R424-6.

51. Malek, T.R., B.O. Porter, and Y.W. He, *Multiple gamma c-dependent cytokines regulate T-cell development.* Immunol Today, 1999. **20**(2): p. 71-6.

52. Kearney, E.R., et al., *Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo.* Immunity, 1994. **1**(4): p. 327-39.

53. Chen, Z.M. and M.K. Jenkins, *Revealing the in vivo behavior of CD4+ T cells specific for an antigen expressed in Escherichia coli.* J Immunol, 1998. **160**(7): p. 3462-70.

54. Ferreira, C., et al., *Differential survival of naive CD4 and CD8 T cells.* J Immunol, 2000. **165**(7): p. 3689-94.

55. Sprent, J., *Burnet oration. T-cell survival and the role of cytokines.* Immunol Cell Biol, 2001. **79**(3): p. 199-206.

56. Tough, D.F. and J. Sprent, *Life span of naive and memory T cells.* Stem Cells, 1995. **13**(3): p. 242-9.

57. Schluns, K.S., et al., *Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo.* Nat Immunol, 2000. **1**(5): p. 426-32.

58. Tanchot, C., et al., *Differential requirements for survival and proliferation of CD8 naive or memory T cells.* Science, 1997. **276**(5321): p. 2057-62.

59. Berzins, S.P., R.L. Boyd, and J.F. Miller, *The role of the thymus and recent thymic migrants in the maintenance of the adult peripheral lymphocyte pool.* J Exp Med, 1998. **187**(11): p. 1839-48.

60. Vella, A.T., et al., *Cytokine-induced survival of activated T cells in vitro and in vivo.* Proc Natl Acad Sci U S A, 1998. **95**(7): p. 3810-5.

61. Van Parijs, L., A. Ibraghimov, and A.K. Abbas, *The roles of costimulation and Fas in T cell apoptosis and peripheral tolerance.* Immunity, 1996. **4**(3): p. 321-8.

62. Sytwu, H.K., R.S. Liblau, and H.O. McDevitt, *The roles of Fas/APO-1 (CD95) and TNF in antigen-induced programmed cell death in T cell receptor transgenic mice.* Immunity, 1996. **5**(1): p. 17-30.

63. Lenardo, M.J., *Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis.* Nature, 1991. **353**(6347): p. 858-61.

64. Sprent, J., *T and B memory cells.* Cell, 1994. **76**(2): p. 315-22.

65. Pullen, A.M., et al., *Surprisingly uneven distribution of the T cell receptor V beta repertoire in wild mice.* J Exp Med, 1990. **171**(1): p. 49-62.

66. Marrack, P., et al., *Homeostasis of alpha beta TCR+ T cells.* Nat Immunol, 2000. **1**(2): p. 107-11.

67. Rocha, B., N. Dautigny, and P. Pereira, *Peripheral T lymphocytes: expansion potential and homeostatic regulation of pool sizes and CD4/CD8 ratios in vivo.* Eur J Immunol, 1989. **19**(5): p. 905-11.

68. Beutner, U. and H.R. MacDonald, *TCR-MHC class II interaction is required for peripheral expansion of CD4 cells in a T cell-deficient host.* Int Immunol, 1998. **10**(3): p. 305-10.

69. Ernst, B., et al., *The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery.* Immunity, 1999. **11**(2): p. 173-81.

70. Goldrath, A.W. and M.J. Bevan, *Low-affinity ligands for the TCR drive proliferation of mature CD8+ T cells in lymphopenic hosts.* Immunity, 1999. **11**(2): p. 183-90.

71. Kirberg, J., A. Berns, and H. von Boehmer, *Peripheral T cell survival requires continual ligation of the T cell receptor to major histocompatibility complex-encoded molecules.* J Exp Med, 1997. **186**(8): p. 1269-75.

72. Brocker, T., *Survival of mature CD4 T lymphocytes is dependent on major histocompatibility complex class II-expressing dendritic cells.* J Exp Med, 1997. **186**(8): p. 1223-32.

73. Tan, J.T., et al., *IL-7 is critical for homeostatic proliferation and survival of naive T cells.* Proc Natl Acad Sci U S A, 2001. **98**(15): p. 8732-7.

74. Tan, J.T., et al., *Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells.* J Exp Med, 2002. **195**(12): p. 1523-32.

75. Vivien, L., C. Benoist, and D. Mathis, *T lymphocytes need IL-7 but not IL-4 or IL-6 to survive in vivo.* Int Immunol, 2001. **13**(6): p. 763-8.

76. von Boehmer, H. and K. Hafen, *The life span of naive alpha/beta T cells in secondary lymphoid organs.* J Exp Med, 1993. **177**(4): p. 891-6.

77. Viret, C., F.S. Wong, and C.A. Janeway, Jr., *Designing and maintaining the mature TCR repertoire: the continuum of self-peptide:self-MHC complex recognition.* Immunity, 1999. **10**(5): p. 559-68.

78. Kieper, W.C. and S.C. Jameson, *Homeostatic expansion and phenotypic conversion of naive T cells in response to self peptide/MHC ligands.* Proc Natl Acad Sci U S A, 1999. **96**(23): p. 13306-11.

79. Swain, S.L., H. Hu, and G. Huston, *Class II-independent generation of CD4 memory T cells from effectors.* Science, 1999. **286**(5443): p. 1381-3.

80. Murali-Krishna, K., et al., *Persistence of memory CD8 T cells in MHC class I-deficient mice.* Science, 1999. **286**(5443): p. 1377-81.

81. Jameson, S.C., *Maintaining the norm: T-cell homeostasis.* Nat Rev Immunol, 2002. **2**(8): p. 547-56.

82. Geiselhart, L.A., et al., *IL-7 administration alters the CD4:CD8 ratio, increases T cell numbers, and increases T cell function in the absence of activation.* J Immunol, 2001. **166**(5): p. 3019-27.

83. Kieper, W.C., et al., *Overexpression of interleukin (IL)-7 leads to IL-15-independent generation of memory phenotype CD8+ T cells.* J Exp Med, 2002. **195**(12): p. 1533-9.

84. Fry, T.J., et al., *A potential role for interleukin-7 in T-cell homeostasis.* Blood, 2001. **97**(10): p. 2983-90.

85. Napolitano, L.A., et al., *Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis.* Nat Med, 2001. **7**(1): p. 73-9.

86. Dummer, W., et al., *Autologous regulation of naive T cell homeostasis within the T cell compartment.* J Immunol, 2001. **166**(4): p. 2460-8.

87. Plunkett, F.J., et al., *Regulation of apoptosis and replicative senescence in CD8+ T cell following acute viral infection.* Apoptosis, 2000. **5**(5): p. 431-4.

88. Friedl, P. and M. Gunzer, *Interaction of T cells with APCs: the serial encounter model.* Trends Immunol, 2001. **22**(4): p. 187-91.

89. Frei, P.C., B. Benacerraf, and G.J. Thorbecke, *Phagocytosis of the Antigen, a Crucial Step in the Induction of the Primary Response.* Proc Natl Acad Sci U S A, 1965. **53**: p. 20-3.

90. Ingulli, E., et al., *In vivo detection of dendritic cell antigen presentation to CD4(+) T cells.* J Exp Med, 1997. **185**(12): p. 2133-41.

91. Mueller, D.L., M.K. Jenkins, and R.H. Schwartz, *Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the*

outcome of T cell antigen receptor occupancy. Annu Rev Immunol, 1989. **7**: p. 445-80.

92. Linsley, P.S. and J.A. Ledbetter, *The role of the CD28 receptor during T cell responses to antigen.* Annu Rev Immunol, 1993. **11**: p. 191-212.

93. Ledbetter, J.A., et al., *CD28 ligation in T-cell activation: evidence for two signal transduction pathways.* Blood, 1990. **75**(7): p. 1531-9.

94. Geppert, T.D. and P.E. Lipsky, *Activation of T lymphocytes by immobilized monoclonal antibodies to CD3. Regulatory influences of monoclonal antibodies to additional T cell surface determinants.* J Clin Invest, 1988. **81**(5): p. 1497-505.

95. Janeway, C.A., Jr. and K. Bottomly, *Signals and signs for lymphocyte responses.* Cell, 1994. **76**(2): p. 275-85.

96. Chan, A.C. and A.S. Shaw, *Regulation of antigen receptor signal transduction by protein tyrosine kinases.* Curr Opin Immunol, 1996. **8**(3): p. 394-401.

97. Pape, K.A., et al., *Use of adoptive transfer of T-cell-antigen-receptor-transgenic T cell for the study of T-cell activation in vivo.* Immunol Rev, 1997. **156**: p. 67-78.

98. Carreno, B.M. and M. Collins, *The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses.* Annu Rev Immunol, 2002. **20**: p. 29-53.

99. Weinberg, A.D., A.T. Vella, and M. Croft, *OX-40: life beyond the effector T cell stage.* Semin Immunol, 1998. **10**(6): p. 471-80.

100. Tivol, E.A., et al., *Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4.* Immunity, 1995. **3**(5): p. 541-7.

101. Chambers, C.A. and J.P. Allison, *Costimulatory regulation of T cell function.* Curr Opin Cell Biol, 1999. **11**(2): p. 203-10.

102. Conze, D., et al., *c-Jun NH(2)-terminal kinase (JNK)1 and JNK2 have distinct roles in CD8(+) T cell activation.* J Exp Med, 2002. **195**(7): p. 811-23.

103. Jung, T.M., et al., *Down-regulation of homing receptors after T cell activation.* J Immunol, 1988. **141**(12): p. 4110-7.

104. Tough, D.F., et al., *Stimulation of naive and memory T cells by cytokines.* Immunol Rev, 1999. **170**: p. 39-47.

105. Bradley, L.M., et al., *Characterization of antigen-specific CD4+ effector T cells in vivo: immunization results in a transient population of MEL-14-, CD45RB- helper cells that secretes interleukin 2 (IL-2), IL-3, IL-4, and interferon gamma.* J Exp Med, 1991. **174**(3): p. 547-59.

106. Granger, G.A. and W.P. Kolb, *Lymphocyte in vitro cytotoxicity: mechanisms of immune and non-immune small lymphocyte mediated target L cell destruction.* J Immunol, 1968. **101**(1): p. 111-20.

107. Liu, C.C., et al., *Identification, isolation, and characterization of a novel cytotoxin in murine cytolytic lymphocytes.* Cell, 1987. **51**(3): p. 393-403.

108. Redelman, D. and D. Hudig, *The mechanism of cell-mediated cytotoxicity. I. Killing by murine cytotoxic T lymphocytes requires cell surface thiols and activated proteases.* J Immunol, 1980. **124**(2): p. 870-8.

109. Shahinian, A., et al., *Differential T cell costimulatory requirements in CD28-deficient mice.* Science, 1993. **261**(5121): p. 609-12.

110. Boulougouris, G., et al., *IL-2-independent activation and proliferation in human T cells induced by CD28.* J Immunol, 1999. **163**(4): p. 1809-16.

111. Harding, F.A., et al., *CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones.* Nature, 1992. **356**(6370): p. 607-9.

112. Seder, R.A. and W.E. Paul, *Acquisition of lymphokine-producing phenotype by CD4+ T cells.* Annu Rev Immunol, 1994. **12**: p. 635-73.

113. Su, H.C., et al., *CD4+ and CD8+ T cell interactions in IFN-gamma and IL-4 responses to viral infections: requirements for IL-2.* J Immunol, 1998. **160**(10): p. 5007-17.

114. Price, D.A., et al., *Cytotoxic T lymphocytes, chemokines and antiviral immunity.* Immunol Today, 1999. **20**(5): p. 212-6.

115. Kagi, D., et al., *Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo.* Annu Rev Immunol, 1996. **14**: p. 207-32.

116. van den Broek, M.F., et al., *Immune defence in mice lacking type I and/or type II interferon receptors.* Immunol Rev, 1995. **148**: p. 5-18.

117. Van Parijs, L., A. Biuckians, and A.K. Abbas, *Functional roles of Fas and Bcl-2-regulated apoptosis of T lymphocytes.* J Immunol, 1998. **160**(5): p. 2065-71.

118. Van Parijs, L. and A.K. Abbas, *Role of Fas-mediated cell death in the regulation of immune responses.* Curr Opin Immunol, 1996. **8**(3): p. 355-61.

119. Kagi, D., et al., *Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity.* Science, 1994. **265**(5171): p. 528-30.

120. Walsh, C.M., et al., *Immune function in mice lacking the perforin gene.* Proc Natl Acad Sci U S A, 1994. **91**(23): p. 10854-8.

121. Boldin, M.P., et al., *Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death.* Cell, 1996. **85**(6): p. 803-15.

122. Muzio, M., et al., *FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex.* Cell, 1996. **85**(6): p. 817-27.

123. Willerford, D.M., et al., *Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment.* Immunity, 1995. **3**(4): p. 521-30.

124. Wang, R., et al., *Induction of sensitivity to activation-induced death in primary CD4+ cells: a role for interleukin-2 in the negative regulation of responses by mature CD4+ T cells.* Eur J Immunol, 1996. **26**(9): p. 2263-70.

125. Zheng, L., et al., *T cell growth cytokines cause the superinduction of molecules mediating antigen-induced T lymphocyte death.* J Immunol, 1998. **160**(2): p. 763-9.

126. Lord, J.D., et al., *The IL-2 receptor promotes proliferation, bcl-2 and bcl-x induction, but not cell viability through the adapter molecule Shc.* J Immunol, 1998. **161**(9): p. 4627-33.

127. Miyazaki, T., et al., *Three distinct IL-2 signaling pathways mediated by bcl-2, c-myc, and lck cooperate in hematopoietic cell proliferation.* Cell, 1995. **81**(2): p. 223-31.

128. Sadlack, B., et al., *Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene.* Cell, 1993. **75**(2): p. 253-61.

129. Suzuki, H., et al., *Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor beta.* Science, 1995. **268**(5216): p. 1472-6.

130. Van Parijs, L., et al., *Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas-mediated activation-induced cell death.* Immunity, 1999. **11**(3): p. 281-8.

131. Refaeli, Y., et al., *Biochemical mechanisms of IL-2-regulated Fas-mediated T cell apoptosis.* Immunity, 1998. **8**(5): p. 615-23.

132. Almeida, A.R., et al., *Homeostasis of peripheral CD4+ T cells: IL-2R alpha and IL-2 shape a population of regulatory cells that controls CD4+ T cell numbers.* J Immunol, 2002. **169**(9): p. 4850-60.

133. Antov, A., et al., *Essential role for STAT5 signaling in CD25+CD4+ regulatory T cell homeostasis and the maintenance of self-tolerance.* J Immunol, 2003. **171**(7): p. 3435-41.

134. Gupta, S., *A decision between life and death during TNF-alpha-induced signaling.* J Clin Immunol, 2002. **22**(4): p. 185-94.

135. Hildeman, D.A., et al., *Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim.* Immunity, 2002. **16**(6): p. 759-67.

136. Deng, G. and E.R. Podack, *Suppression of apoptosis in a cytotoxic T-cell line by interleukin 2-mediated gene transcription and deregulated expression of the protooncogene bcl-2.* Proc Natl Acad Sci U S A, 1993. **90**(6): p. 2189-93.

137. Ihle, J.N., *Cytokine receptor signalling.* Nature, 1995. **377**(6550): p. 591-4.

138. O'Shea, J.J., *Jaks, STATs, cytokine signal transduction, and immunoregulation: are we there yet?* Immunity, 1997. **7**(1): p. 1-11.

139. Horvath, C.M. and J.E. Darnell, *The state of the STATs: recent developments in the study of signal transduction to the nucleus.* Curr Opin Cell Biol, 1997. **9**(2): p. 233-9.

140. Imada, K. and W.J. Leonard, *The Jak-STAT pathway.* Mol Immunol, 2000. **37**(1-2): p. 1-11.

141. Velazquez, L., et al., *Distinct domains of the protein tyrosine kinase tyk2 required for binding of interferon-alpha/beta and for signal transduction.* J Biol Chem, 1995. **270**(7): p. 3327-34.

142. Fujitani, Y., et al., *An alternative pathway for STAT activation that is mediated by the direct interaction between JAK and STAT.* Oncogene, 1997. **14**(7): p. 751-61.

143. Frank, S.J., et al., *Interaction of the growth hormone receptor cytoplasmic domain with the JAK2 tyrosine kinase.* Endocrinology, 1994. **135**(5): p. 2228-39.

144. Chen, M., et al., *The amino terminus of JAK3 is necessary and sufficient for binding to the common gamma chain and confers the ability to transmit interleukin 2-mediated signals.* Proc Natl Acad Sci U S A, 1997. **94**(13): p. 6910-5.

145. Frank, S.J., et al., *Regions of the JAK2 tyrosine kinase required for coupling to the growth hormone receptor.* J Biol Chem, 1995. **270**(24): p. 14776-85.

146. Kohlhuber, F., et al., *A JAK1/JAK2 chimera can sustain alpha and gamma interferon responses.* Mol Cell Biol, 1997. **17**(2): p. 695-706.

147. O'Shea, J.J., et al., *Advances in the understanding of cytokine signal transduction: the role of Jaks and STATs in immunoregulation and the pathogenesis of immunodeficiency.* J Clin Immunol, 1997. **17**(6): p. 431-47.

148. Muller, M., et al., *The protein tyrosine kinase JAK1 complements defects in interferon-alpha/beta and -gamma signal transduction.* Nature, 1993. **366**(6451): p. 129-35.

149. Velazquez, L., et al., *A protein tyrosine kinase in the interferon alpha/beta signaling pathway.* Cell, 1992. **70**(2): p. 313-22.

150. Russell, S.M., et al., *Interaction of IL-2R beta and gamma c chains with Jak1 and Jak3: implications for XSCID and XCID.* Science, 1994. **266**(5187): p. 1042-5.

151. Miyazaki, T., et al., *Functional activation of Jak1 and Jak3 by selective association with IL-2 receptor subunits.* Science, 1994. **266**(5187): p. 1045-7.

152. Quelle, F.W., et al., *JAK2 associates with the beta c chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region.* Mol Cell Biol, 1994. **14**(7): p. 4335-41.

153. Johnston, J.A., et al., *Phosphorylation and activation of the Jak-3 Janus kinase in response to interleukin-2.* Nature, 1994. **370**(6485): p. 151-3.

154. Darnell, J.E., Jr., *STATs and gene regulation.* Science, 1997. **277**(5332): p. 1630-5.

155. Ward, A.C., I. Touw, and A. Yoshimura, *The Jak-Stat pathway in normal and perturbed hematopoiesis.* Blood, 2000. **95**(1): p. 19-29.

156. Seidel, H.M., et al., *Spacing of palindromic half sites as a determinant of selective STAT (signal transducers and activators of transcription) DNA binding and transcriptional activity.* Proc Natl Acad Sci U S A, 1995. **92**(7): p. 3041-5.

157. Cacalano, N.A., et al., *Autosomal SCID caused by a point mutation in the N-terminus of Jak3: mapping of the Jak3-receptor interaction domain.* Embo J, 1999. **18**(6): p. 1549-58.

158. Stahl, N., et al., *Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors.* Science, 1995. **267**(5202): p. 1349-53.

159. Greenlund, A.C., et al., *Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91).* Embo J, 1994. **13**(7): p. 1591-600.

160. Shuai, K., et al., *Interferon activation of the transcription factor Stat91 involves dimerization through SH2-phosphotyrosyl peptide interactions.* Cell, 1994. **76**(5): p. 821-8.

161. Gupta, S., et al., *The SH2 domains of Stat1 and Stat2 mediate multiple interactions in the transduction of IFN-alpha signals.* Embo J, 1996. **15**(5): p. 1075-84.

162. Ota, N., et al., *N-domain-dependent nonphosphorylated STAT4 dimers required for cytokine-driven activation.* Nat Immunol, 2004. **5**(2): p. 208-15.

163. Leonard, W.J., *Role of Jak kinases and STATs in cytokine signal transduction.* Int J Hematol, 2001. **73**(3): p. 271-7.

164. Winston, L.A. and T. Hunter, *Intracellular signalling: putting JAKs on the kinase MAP.* Curr Biol, 1996. **6**(6): p. 668-71.

165. Han, Y., et al., *Participation of JAK and STAT proteins in growth hormone-induced signaling.* J Biol Chem, 1996. **271**(10): p. 5947-52.

166. Ihle, J.N. and I.M. Kerr, *Jaks and Stats in signaling by the cytokine receptor superfamily.* Trends Genet, 1995. **11**(2): p. 69-74.

167. Rane, S.G. and E.P. Reddy, *Janus kinases: components of multiple signaling pathways.* Oncogene, 2000. **19**(49): p. 5662-79.

168. Al-Shami, A. and P.H. Naccache, *Granulocyte-macrophage colony-stimulating factor-activated signaling pathways in human neutrophils. Involvement of Jak2 in the stimulation of phosphatidylinositol 3-kinase.* J Biol Chem, 1999. **274**(9): p. 5333-8.

169. Kim, T.K. and T. Maniatis, *Regulation of interferon-gamma-activated STAT1 by the ubiquitin-proteasome pathway.* Science, 1996. **273**(5282): p. 1717-9.

170. Endo, T.A., et al., *A new protein containing an SH2 domain that inhibits JAK kinases.* Nature, 1997. **387**(6636): p. 921-4.

171. Kirkpatrick, D.T. and T.D. Petes, *Repair of DNA loops involves DNA-mismatch and nucleotide-excision repair proteins.* Nature, 1997. **387**(6636): p. 929-31.

172. Naka, T., et al., *Structure and function of a new STAT-induced STAT inhibitor.* Nature, 1997. **387**(6636): p. 924-9.

173. Starr, R., et al., *A family of cytokine-inducible inhibitors of signalling.* Nature, 1997. **387**(6636): p. 917-21.

174. Yasukawa, H., et al., *The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop.* Embo J, 1999. **18**(5): p. 1309-20.

175. Rui, H., et al., *Activation of the Jak2-Stat5 signaling pathway in Nb2 lymphoma cells by an anti-apoptotic agent, aurintricarboxylic acid.* J Biol Chem, 1998. **273**(1): p. 28-32.

176. You, M., D.H. Yu, and G.S. Feng, *Shp-2 tyrosine phosphatase functions as a negative regulator of the interferon-stimulated Jak/STAT pathway.* Mol Cell Biol, 1999. **19**(3): p. 2416-24.

177. Kirken, R.A., et al., *Identification of interleukin-2 receptor-associated tyrosine kinase p116 as novel leukocyte-specific Janus kinase.* J Biol Chem, 1994. **269**(29): p. 19136-41.

178. Asao, H., et al., *Cutting edge: the common gamma-chain is an indispensable subunit of the IL-21 receptor complex.* J Immunol, 2001. **167**(1): p. 1-5.

179. Thomis, D.C., et al., *Defects in B lymphocyte maturation and T lymphocyte activation in mice lacking Jak3.* Science, 1995. **270**(5237): p. 794-7.

180. Nosaka, T., et al., *Defective lymphoid development in mice lacking Jak3.* Science, 1995. **270**(5237): p. 800-2.

181. Park, S.Y., et al., *Developmental defects of lymphoid cells in Jak3 kinase-deficient mice.* Immunity, 1995. **3**(6): p. 771-82.

182. Noguchi, M., et al., *Interleukin-2 receptor gamma chain: a functional component of the interleukin-7 receptor.* Science, 1993. **262**(5141): p. 1877-80.

183. Russell, S.M., et al., *Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development.* Science, 1995. **270**(5237): p. 797-800.

184. Noguchi, M., et al., *Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans.* Cell, 1993. **73**(1): p. 147-57.

185. von Freeden-Jeffry, U., et al., *Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine.* J Exp Med, 1995. **181**(4): p. 1519-26.

186. Peschon, J.J., et al., *Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice.* J Exp Med, 1994. **180**(5): p. 1955-60.

187. von Freeden-Jeffry, U., et al., *The earliest T lineage-committed cells depend on IL-7 for Bcl-2 expression and normal cell cycle progression.* Immunity, 1997. **7**(1): p. 147-54.

188. Moore, T.A., et al., *Inhibition of gamma delta T cell development and early thymocyte maturation in IL-7 -/- mice.* J Immunol, 1996. **157**(6): p. 2366-73.

189. Cao, X., et al., *Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain.* Immunity, 1995. **2**(3): p. 223-38.

190. Ilangumaran, S., et al., *Suppressor of cytokine signaling 1 attenuates IL-15 receptor signaling in CD8+ thymocytes.* Blood, 2003. **102**(12): p. 4115-22.

191. Malissen, M., et al., *The common cytokine receptor gamma chain controls survival of gamma/delta T cells.* J Exp Med, 1997. **186**(8): p. 1277-85.

192. Maki, K., S. Sunaga, and K. Ikuta, *The V-J recombination of T cell receptor-gamma genes is blocked in interleukin-7 receptor-deficient mice.* J Exp Med, 1996. **184**(6): p. 2423-7.

193. Leclercq, G., et al., *Differential effects of interleukin-15 and interleukin-2 on differentiation of bipotential T/natural killer progenitor cells.* J Exp Med, 1996. **184**(2): p. 325-36.

194. Cavazzana-Calvo, M., et al., *Role of interleukin-2 (IL-2), IL-7, and IL-15 in natural killer cell differentiation from cord blood hematopoietic progenitor cells*

and from gamma c transduced severe combined immunodeficiency X1 bone marrow cells. Blood, 1996. **88**(10): p. 3901-9.

195. Witherden, D., et al., *Tetracycline-controllable selection of CD4(+) T cells: half-life and survival signals in the absence of major histocompatibility complex class II molecules.* J Exp Med, 2000. **191**(2): p. 355-64.

196. Seddon, B. and R. Zamoyska, *TCR and IL-7 receptor signals can operate independently or synergize to promote lymphopenia-induced expansion of naive T cells.* J Immunol, 2002. **169**(7): p. 3752-9.

197. Lodolce, J.P., et al., *IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation.* Immunity, 1998. **9**(5): p. 669-76.

198. Zhang, X., et al., *Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15.* Immunity, 1998. **8**(5): p. 591-9.

199. Kennedy, M.K., et al., *Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice.* J Exp Med, 2000. **191**(5): p. 771-80.

200. Goldrath, A.W., et al., *Cytokine requirements for acute and Basal homeostatic proliferation of naive and memory CD8+ T cells.* J Exp Med, 2002. **195**(12): p. 1515-22.

201. Kondrack, R.M., et al., *Interleukin 7 regulates the survival and generation of memory CD4 cells.* J Exp Med, 2003. **198**(12): p. 1797-806.

202. Seddon, B., P. Tomlinson, and R. Zamoyska, *Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells.* Nat Immunol, 2003. **4**(7): p. 680-6.

203. Nakajima, H. and W.J. Leonard, *Impaired peripheral deletion of activated T cells in mice lacking the common cytokine receptor gamma-chain: defective Fas ligand expression in gamma-chain-deficient mice.* J Immunol, 1997. **159**(10): p. 4737-44.

204. Thomis, D.C. and L.J. Berg, *The role of Jak3 in lymphoid development, activation, and signaling.* Curr Opin Immunol, 1997. **9**(4): p. 541-7.

205. Thomis, D.C. and L.J. Berg, *Peripheral expression of Jak3 is required to maintain T lymphocyte function.* J Exp Med, 1997. **185**(2): p. 197-206.

206. Lantz, O., et al., *Gamma chain required for naive CD4+ T cell survival but not for antigen proliferation.* Nat Immunol, 2000. **1**(1): p. 54-8.

207. Bachmann, M.F., et al., *Antiviral immune responses in mice deficient for both interleukin-2 and interleukin-4.* J Virol, 1995. **69**(8): p. 4842-6.

208. Yu, A., et al., *Efficient induction of primary and secondary T cell-dependent immune responses in vivo in the absence of functional IL-2 and IL-15 receptors.* J Immunol, 2003. **170**(1): p. 236-42.

209. Kramer, S., et al., *Thymic selection and peptide-induced activation of T cell receptor-transgenic CD8 T cells in interleukin-2-deficient mice.* Eur J Immunol, 1994. **24**(10): p. 2317-22.

210. Cousens, L.P., J.S. Orange, and C.A. Biron, *Endogenous IL-2 contributes to T cell expansion and IFN-gamma production during lymphocytic choriomeningitis virus infection.* J Immunol, 1995. **155**(12): p. 5690-9.

211. D'Souza, W.N. and L. Lefrancois, *IL-2 is not required for the initiation of CD8 T cell cycling but sustains expansion.* J Immunol, 2003. **171**(11): p. 5727-35.

212. Schluns, K.S., et al., *Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells.* J Immunol, 2002. **168**(10): p. 4827-31.

213. Strasser, A., et al., *Bcl-2 expression promotes B- but not T-lymphoid development in scid mice.* Nature, 1994. **368**(6470): p. 457-60.

214. Welsh, R.M., Jr., et al., *Antibody-complement interactions with purified lymphocytic choriomeningitis virus.* Virology, 1976. **73**(1): p. 59-71.

215. Pannetier, C., et al., *The sizes of the CDR3 hypervariable regions of the murine T-cell receptor beta chains vary as a function of the recombined germ-line segments.* Proc Natl Acad Sci U S A, 1993. **90**(9): p. 4319-23.

216. Gudmundsdottir, H., A.D. Wells, and L.A. Turka, *Dynamics and requirements of T cell clonal expansion in vivo at the single-cell level: effector function is linked to proliferative capacity.* J Immunol, 1999. **162**(9): p. 5212-23.

217. Norbury, C.C., et al., *Multiple antigen-specific processing pathways for activating naive CD8+ T cells in vivo.* J Immunol, 2001. **166**(7): p. 4355-62.

218. van der Most, R.G., et al., *Identification of Db- and Kb-restricted subdominant cytotoxic T-cell responses in lymphocytic choriomeningitis virus-infected mice.* Virology, 1998. **240**(1): p. 158-67.

219. Whitton, J.L., P.J. Southern, and M.B. Oldstone, *Analyses of the cytotoxic T lymphocyte responses to glycoprotein and nucleoprotein components of lymphocytic choriomeningitis virus.* Virology, 1988. **162**(2): p. 321-7.

220. Ahmed, R., et al., *Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence.* J Exp Med, 1984. **160**(2): p. 521-40.

221. Webb, L.M., B.M. Foxwell, and M. Feldmann, *Putative role for interleukin-7 in the maintenance of the recirculating naive CD4+ T-cell pool.* Immunology, 1999. **98**(3): p. 400-5.

222. Soares, M.V., et al., *IL-7-dependent extrathymic expansion of CD45RA+ T cells enables preservation of a naive repertoire.* J Immunol, 1998. **161**(11): p. 5909-17.

223. Boursalian, T.E. and K. Bottomly, *Survival of naive CD4 T cells: roles of restricting versus selecting MHC class II and cytokine milieu.* J Immunol, 1999. **162**(7): p. 3795-801.

224. Leonard, W.J., E.W. Shores, and P.E. Love, *Role of the common cytokine receptor gamma chain in cytokine signaling and lymphoid development.* Immunol Rev, 1995. **148**: p. 97-114.

225. Nakajima, H., et al., *The common cytokine receptor gamma chain plays an essential role in regulating lymphoid homeostasis.* J Exp Med, 1997. **185**(2): p. 189-95.

226. Chambers, C.A., et al., *Thymocyte development is normal in CTLA-4-deficient mice.* Proc Natl Acad Sci U S A, 1997. **94**(17): p. 9296-301.

227. Waterhouse, P., et al., *Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4.* Science, 1995. **270**(5238): p. 985-8.

228. Chambers, C.A., et al., *Secondary but not primary T cell responses are enhanced in CTLA-4-deficient CD8+ T cells.* Eur J Immunol, 1998. **28**(10): p. 3137-43.

229. Oosterwegel, M.A., et al., *The role of CTLA-4 in regulating Th2 differentiation.* J Immunol, 1999. **163**(5): p. 2634-9.

230. Gorski, J., et al., *Circulating T cell repertoire complexity in normal individuals and bone marrow recipients analyzed by CDR3 size spectratyping. Correlation with immune status.* J Immunol, 1994. **152**(10): p. 5109-19.

231. Maslanka, K., et al., *Molecular analysis of T cell repertoires. Spectratypes generated by multiplex polymerase chain reaction and evaluated by radioactivity or fluorescence.* Hum Immunol, 1995. **44**(1): p. 28-34.

232. Waterhouse, P., et al., *Normal thymic selection, normal viability and decreased lymphoproliferation in T cell receptor-transgenic CTLA-4-deficient mice.* Eur J Immunol, 1997. **27**(8): p. 1887-92.

233. Saijo, K., et al., *Crucial role of Jak3 in negative selection of self-reactive T cells.* J Exp Med, 1997. **185**(2): p. 351-6.

234. Suda, T., et al., *Expression of the Fas ligand in cells of T cell lineage.* J Immunol, 1995. **154**(8): p. 3806-13.

235. Van Parijs, L., et al., *Autoimmunity as a consequence of retrovirus-mediated expression of C-FLIP in lymphocytes.* Immunity, 1999. **11**(6): p. 763-70.

236. Gill, J., et al., *Thymic generation and regeneration.* Immunol Rev, 2003. **195**: p. 28-50.

237. Godfrey, D.I., A. Zlotnik, and T. Suda, *Phenotypic and functional characterization of c-kit expression during intrathymic T cell development.* J Immunol, 1992. **149**(7): p. 2281-5.

238. Rodewald, H.R., et al., *Pro-thymocyte expansion by c-kit and the common cytokine receptor gamma chain is essential for repertoire formation.* Immunity, 1997. **6**(3): p. 265-72.

239. Kearse, K.P., et al., *Developmental regulation of alpha beta T cell antigen receptor expression results from differential stability of nascent TCR alpha proteins within the endoplasmic reticulum of immature and mature T cells.* Embo J, 1994. **13**(19): p. 4504-14.

240. Wilson, A., H.R. MacDonald, and F. Radtke, *Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus.* J Exp Med, 2001. **194**(7): p. 1003-12.

241. Fehling, H.J., et al., *Crucial role of the pre-T-cell receptor alpha gene in development of alpha beta but not gamma delta T cells.* Nature, 1995. **375**(6534): p. 795-8.

242. Dudley, E.C., et al., *T cell receptor beta chain gene rearrangement and selection during thymocyte development in adult mice.* Immunity, 1994. **1**(2): p. 83-93.

243. Tatsumi, Y., et al., *Differentiation of thymocytes from CD3-CD4-CD8- through CD3-CD4-CD8+ into more mature stages induced by a thymic stromal cell clone.* Proc Natl Acad Sci U S A, 1990. **87**(7): p. 2750-4.

244. von Boehmer, H., et al., *Pleiotropic changes controlled by the pre-T-cell receptor.* Curr Opin Immunol, 1999. **11**(2): p. 135-42.

245. Nossal, G.J., *Negative selection of lymphocytes.* Cell, 1994. **76**(2): p. 229-39.

246. Hawkins, C.J. and D.L. Vaux, *Analysis of the role of bcl-2 in apoptosis.* Immunol Rev, 1994. **142**: p. 127-39.

247. Nakayama, K., et al., *Disappearance of the lymphoid system in Bcl-2 homozygous mutant chimeric mice.* Science, 1993. **261**(5128): p. 1584-8.

248. Akashi, K., et al., *Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice.* Cell, 1997. **89**(7): p. 1033-41.

249. Maraskovsky, E., et al., *Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor-deficient mice but not in mutant rag-1-/- mice.* Cell, 1997. **89**(7): p. 1011-9.

250. Rodewald, H.R., C. Waskow, and C. Haller, *Essential requirement for c-kit and common gamma chain in thymocyte development cannot be overruled by enforced expression of Bcl-2.* J Exp Med, 2001. **193**(12): p. 1431-7.

251. Tanchot, C., et al., *Lymphocyte homeostasis.* Semin Immunol, 1997. **9**(6): p. 331-7.

252. Seddon, B., et al., *Long-term survival but impaired homeostatic proliferation of Naive T cells in the absence of p56lck.* Science, 2000. **290**(5489): p. 127-31.

253. O'Reilly, L.A., A.W. Harris, and A. Strasser, *bcl-2 transgene expression promotes survival and reduces proliferation of CD3-CD4-CD8- T cell progenitors.* Int Immunol, 1997. **9**(9): p. 1291-301.

254. Welsh, R.M., *Lymphocytic Choriomeningitis Virus Model for the Study of Cellular Imm*, in *effects of Microbes on the Immune System*, M.W.C.a.R.S. Fujinami, Editor. 2000, Lippincott Williams & Wilkins: Philadelphia. p. 289-312.

255. Buchmeier, M.J., et al., *The virology and immunobiology of lymphocytic choriomeningitis virus infection.* Adv Immunol, 1980. **30**: p. 275-331.

256. Butz, E.A. and M.J. Bevan, *Massive expansion of antigen-specific CD8+ T cells during an acute virus infection.* Immunity, 1998. **8**(2): p. 167-75.

257. Homann, D., L. Teyton, and M.B. Oldstone, *Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory.* Nat Med, 2001. **7**(8): p. 913-9.

258. Kagi, D., et al., *The roles of perforin- and Fas-dependent cytotoxicity in protection against cytopathic and noncytopathic viruses.* Eur J Immunol, 1995. **25**(12): p. 3256-62.

259. Simon, M.M., et al., *In vitro- and ex vivo-derived cytolytic leukocytes from granzyme A x B double knockout mice are defective in granule-mediated apoptosis but not lysis of target cells.* J Exp Med, 1997. **186**(10): p. 1781-6.

260. Whitton, J.L., et al., *Molecular analyses of a five-amino-acid cytotoxic T-lymphocyte (CTL) epitope: an immunodominant region which induces nonreciprocal CTL cross-reactivity.* J Virol, 1989. **63**(10): p. 4303-10.

261. Whitton, J.L., et al., *Molecular definition of a major cytotoxic T-lymphocyte epitope in the glycoprotein of lymphocytic choriomeningitis virus.* J Virol, 1988. **62**(3): p. 687-95.

262. Hudrisier, D., M.B. Oldstone, and J.E. Gairin, *The signal sequence of lymphocytic choriomeningitis virus contains an immunodominant cytotoxic T cell epitope that is restricted by both H-2D(b) and H-2K(b) molecules.* Virology, 1997. **234**(1): p. 62-73.

263. De Boer, R.J., et al., *Recruitment times, proliferation, and apoptosis rates during the CD8(+) T-cell response to lymphocytic choriomeningitis virus.* J Virol, 2001. **75**(22): p. 10663-9.

264. Welsh, R.M., L.K. Selin, and E.S. Razvi, *Role of apoptosis in the regulation of virus-induced T cell responses, immune suppression, and memory.* J Cell Biochem, 1995. **59**(2): p. 135-42.

265. Gozalo-Sanmillan, S., et al., *Cutting edge: two distinct mechanisms lead to impaired T cell homeostasis in Janus kinase 3- and CTLA-4-deficient mice.* J Immunol, 2001. **166**(2): p. 727-30.

266. Leist, T.P., et al., *Functional analysis of T lymphocyte subsets in antiviral host defense.* J Immunol, 1987. **138**(7): p. 2278-81.

267. Moskophidis, D., et al., *Mechanism of recovery from acute virus infection: treatment of lymphocytic choriomeningitis virus-infected mice with monoclonal antibodies reveals that Lyt-2+ T lymphocytes mediate clearance of virus and regulate the antiviral antibody response.* J Virol, 1987. **61**(6): p. 1867-74.

268. Murali-Krishna, K., et al., *Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection.* Immunity, 1998. **8**(2): p. 177-87.

269. McNally, J.M., et al., *Attrition of bystander CD8 T cells during virus-induced T-cell and interferon responses.* J Virol, 2001. **75**(13): p. 5965-76.

270. Wherry, E.J., et al., *Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment.* J Virol, 2003. **77**(8): p. 4911-27.

271. Binder, D., et al., *Virus-induced transient bone marrow aplasia: major role of interferon-alpha/beta during acute infection with the noncytopathic lymphocytic choriomeningitis virus.* J Exp Med, 1997. **185**(3): p. 517-30.

272. Kasaian, M.T., et al., *IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity.* Immunity, 2002. **16**(4): p. 559-69.

273. Thomis, D.C., W. Lee, and L.J. Berg, *T cells from Jak3-deficient mice have intact TCR signaling, but increased apoptosis.* J Immunol, 1997. **159**(10): p. 4708-19.

274. Salazar-Mather, T.P. and K.L. Hokeness, *Calling in the troops: regulation of inflammatory cell trafficking through innate cytokine/chemokine networks.* Viral Immunol, 2003. **16**(3): p. 291-306.

275. Luster, A.D., *The role of chemokines in linking innate and adaptive immunity.* Curr Opin Immunol, 2002. **14**(1): p. 129-35.

276. Di Carlo, E., et al., *IL-21 induces tumor rejection by specific CTL and IFN-gamma-dependent CXC chemokines in syngeneic mice.* J Immunol, 2004. **172**(3): p. 1540-7.

277. Biron, C.A., et al., *Natural killer cells in antiviral defense: function and regulation by innate cytokines.* Annu Rev Immunol, 1999. **17**: p. 189-220.

278. Trinchieri, G., *Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes.* Blood, 1994. **84**(12): p. 4008-27.

279. Wan, Y., et al., *Dendritic cell-derived IL-12 is not required for the generation of cytotoxic, IFN-gamma-secreting, CD8(+) CTL in vivo.* J Immunol, 2001. **167**(9): p. 5027-33.